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CII. THE ENZYMIC PRODUCTION OF VOLATILE PRODUCTS FROM NICOTINE UNDER THE INFLUENCE OF TOBACCO-LEAF EXTRACTS.

(A REPLY TO A. FAITELOWITZ.)

By ANDOR FODOR and ADOLF REIFENBERG.

From the Institute of Biological and Colloidal Chemistry of the Hebrew University, Jerusalem.

(*Received April 6th, 1927.*)

A paper has been published in this *Journal* by Faitelowitz [1927] dealing with experiments from which it may be concluded that mixtures of fermented cigarette-tobacco with water are infected by bacteria in the open air; at the same time an alkaline reaction develops, which is not the case if chloroform is applied. Probably no one will doubt that these facts are correct.

We wish to point out, however, that in our experiments [1925] the macerates were prepared "under the usual conditions of sterility" (p. 830), *i.e.* by using toluene and thymol, and, nevertheless, decomposed nicotine.

The formation of acids during tobacco fermentation was not denied by us; on the contrary the formation of carbonic acid during the decomposition of nicotine has been noted (p. 831).

Since the publication of our paper in this *Journal* we have reported on many additional experiments with regard to the question of tobacco fermentation [1926]. We found a strong parallelism between the decomposition of nicotine and the amount of oxygen absorbed by moistened tobacco leaves, which is a further argument in support of our view that the reaction is one of oxidation. Furthermore, we found an oxygen absorption even after filtering the macerates through porous clay filters or after adding germicides, *e.g.* vucine, mercuric chloride, and even potassium cyanide. Neither the respiration, nor the decomposition of nicotine connected with it, is in any way associated with bacterial processes. Our views on the sum-total of the fermentation process are presented in the above-mentioned paper.

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CIII. THE INTERACTION OF A FINELY DIVIDED LEAD SUSPENSION WITH BLOOD-SERUM, RINGER SOLUTION, AND AQUEOUS PHOSPHATE SOLUTION.[1]

By JOHN BROOKS.

From the Muspratt Laboratory of Physical Chemistry and Electrochemistry, University of Liverpool.

(*Received May 17th, 1927.*)

The present paper deals with the changes which take place when lead in a fine state of subdivision in a stabilising medium (as used in the treatment of malignant disease) is added to blood-serum.

The chief points on which information was desired were:

(i) The possible production of the toxic ionic lead after injection of the lead suspension into the blood-stream.

(ii) The chemical reactions between the constituents of the suspension and those of the blood.

In the literature dealing with lead poisoning there are several references to the form which lead compounds assume in the blood, but no very definite conclusions have been reached.

The question has been investigated by Dr M. Jowett in this laboratory from the standpoint of the solubility of inorganic lead salts in water and in serum respectively. Of these salts the most important are the tertiary and secondary phosphates and the carbonate. Jowett has calculated that the tertiary phosphate $Pb_3(PO_4)_2$ is, under body conditions, the least soluble. Lead salts when introduced into the blood should therefore ultimately pass into this compound since there are phosphate ions present in serum. This has been suggested by Fairhall *et al.* [1926].

Though the transformation into the phosphate would probably be rapid when soluble lead salts were introduced into the blood, the same might not be the case when the lead was in a colloidal form. Various measurements of the rate of combination have therefore been made.

Because of the complexity of both lead suspension and serum some work has also been done with aqueous solutions of sodium phosphate and with a modified Locke-Ringer solution.

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[1] This investigation was undertaken on behalf of the Liverpool Medical Research Organisation.

EXPERIMENTAL.

The lead suspension. This is prepared by Bredig's electrical sparking method using lead electrodes in a 0·5 % gelatin solution containing calcium chloride (*N*/200) as stabilising medium. The average diameter of the particles in the black suspension after centrifuging is 0·2μ, these particles being negatively charged. The freshly-prepared suspension contains approximately 0·5 % total lead; a typical suspension had the composition: 0·51 g. total Pb per 100 cc. This contains

65·6 % Pb as metallic lead,
8·9 % Pb as lead carbonate,
25·5 % Pb as lead hydroxide[1] (by difference).

The concentration of ionic lead (determined by colorimetric estimation of lead in the ultra-filtrate from the suspension) is *ca.* 0·009 %. This corresponds approximately with the value expected from the solubility of lead hydroxide[2]. The chemical composition of the particles varies with the time and extent of exposure of the suspension to the atmosphere, and the suspension eventually turns white on complete oxidation of the lead to lead hydroxide. The total lead content of each fresh suspension used was estimated by the iodometric method described by Fairhall *et al.* [1926].

The aqueous phosphate solution. The solution used had the following composition: 0·00156 *M* Na_2HPO_4, 0·00095 *M* HCl, 0·9 g. NaCl per 100 cc. The p_H of this solution was 7·4 and 1 cc. contained 0·0484 mg. phosphorus, which is within the range of the inorganic phosphorus content of blood-serum.

Ringer solution[3]. This solution was prepared by mixing isotonic solutions of the various salts and had the following composition:

Salt	Moles per litre
NaCl	0·137
KCl	0·00280
$CaCl_2$	0·00197
$NaHCO_3$	0·0264 (unless otherwise stated)
Na_2HPO_4 KH_2PO_4	0·00176 ,, ,,

This solution is used in conjunction with a gas mixture of 95 % oxygen and 5 % carbon dioxide. When saturated with this mixture the p_H at 37·5° is 7·49. The p_H is given by the Henderson-Hasselbalch equation [1917]

$$p_H = p_K + \log \delta + \log \frac{[NaHCO_3]}{[CO_2]},$$

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[1] For the sake of convenience the compound formed by the oxidation of metallic lead in contact with water is called lead hydroxide and written $Pb(OH)_2$, although according to Pleissner [1907] it has the composition $Pb_3O_2(OH)_2$; Glasstone [1922] gives a more complex composition to this substance.

[2] Pleissner [1907] gives 100·5 mg. PbO per litre at 18° for the solubility of lead hydroxide in water.

[3] This solution (with the exception of the phosphate present) as also the method of its p_H regulation is similar to that used by Warburg [1924] in his work on glycolysis.

where $[NaHCO_3]$ and $[CO_2]$ are the concentrations in moles per litre of (sodium) bicarbonate and dissolved carbon dioxide respectively. In the present case at 37·5°

$$[CO_2] = 1{\cdot}18 \times 10^{-3},$$
$$[NaHCO_3] = 2{\cdot}64 \times 10^{-2},$$

whence

$$p_H = 7{\cdot}49,$$
$$p_K + \log \delta = 6{\cdot}14.$$

Serum. Fresh cow's serum was used, thymol being added as preservative. In order to maintain a physiological p_H the serum was saturated with the gas mixture referred to above and subsequent operations were conducted in presence of this mixture. The p_H was then known when the bicarbonate concentration of the serum was determined.

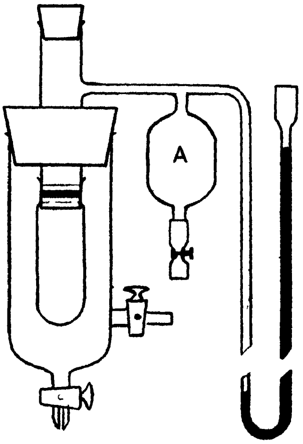

Fig. 1. Apparatus for ultra-filtration.

When the lead suspension was added to serum or to the other two solutions mentioned above there was usually no immediate aggregation of the particles. To estimate the amount of phosphate ion which had disappeared from solution it was necessary to remove the suspended particles. This was done by ultra-filtration under a pressure of 200–300 mm. Hg through collodion bags (prepared from a 6 % collodion solution). The arrangement represented in Fig. 1 permitted ultra-filtration in an atmosphere of the gas mixture and also at different temperatures. The collodion bag was secured to the glass tube by a strip of parchment and tightly-wound linen thread. The air space *A* was necessary to prevent a too rapid fall of pressure during filtration. If necessary the air was displaced by the gas mixture from the apparatus. The first 1·5 cc. of filtrate were rejected. With serum the rate of filtration was 3–5 cc. per hour, with Ringer solution this rate was approximately doubled.

Inorganic phosphorus was estimated by Briggs' modification [1924] of the Bell-Doisy colorimetric method. Bicarbonate concentrations were determined (at 37·5° and in presence of the gas mixture) with Warburg's apparatus and technique [Negelein, 1925].

The experimental procedure was as follows. The lead suspension was added to serum or to Ringer solution previously saturated with gas mixture in such amounts that excess phosphate was present. The gas mixture was bubbled through the liquid for 3–4 minutes, ultra-filtered after a given time, and the uncombined phosphate in 1–3 cc. of the filtrate estimated. The gas mixture was not used with the aqueous phosphate solution.

The combination of the metallic lead present with phosphate is necessarily preceded by its transformation into the hydroxide[1]. Rough measurements had previously shown that the rate of this change in the suspension itself when exposed to air is dependent on the rate of stirring and the area of the air-liquid interface. The reaction is rapid given a sufficient supply of oxygen—as can be seen by the immediate whitening which takes place on adding the suspension to large enough volumes of water. With the amounts of suspension and serum, etc., used in the present experiments, the reaction was complete in less than 5 minutes[2].

Table I gives the results obtained when the lead suspension was added to aqueous phosphate solution. The mixtures were allowed to stand for 7 days before being filtered. The p_H of the mixtures varied from 8·2 to 8·8[3].

Table I. *Lead suspension contains* 0·517 % *total lead.*

cc. phosphate solution	cc. suspension	g. atoms phosphorus bound to lead × 10^4	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$
80	1	0·156	0·62
40	1	0·158	0·63
40	2	0·321	0·64
40	3	0·470	0·63

The last column gives the ratio of g. atoms of phosphorus, which as phosphate had disappeared from solution, to g. atoms of total lead present. The value for the ratio does not differ greatly from that for tertiary lead phosphate, $Pb_3(PO_4)_2$, *i.e.* 0·67. The similarity of the results at different final concentrations of phosphate and also the large amounts of the latter which have disappeared do not favour the view that the disappearance is due to adsorption alone. It seems probable therefore that the particles are converted into particles of lead phosphate.

Before the interaction of the lead suspension with serum was investigated, a few filtration experiments with serum alone were carried out. The ultrafiltrate was colourless and protein-free and contained the same concentration

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[1] Sackur [1905] has shown that pure lead is only attacked by dilute acids in the presence of oxygen. The velocity of solution is practically independent of the strength and concentration of the acid but dependent on the concentration of dissolved oxygen and the rate of stirring.

[2] Measured approximately by a manometric method.

[3] The change of p_H in the very weakly buffered system is apparently due to the following reaction:

$$2HPO_4'' + 3Pb(OH)_2 \rightarrow Pb_3(PO_4)_2 + 2H_2O + 4OH'.$$

of bicarbonate and inorganic phosphorus as the serum itself. Further, the concentration of inorganic phosphorus in the filtrate was unaltered after the serum had stood for 14 days.

Table II summarises the results obtained on adding the suspension to phosphate and Ringer solution and to serum. For comparison are added the values obtained when a solution of lead acetate (5 mg. Pb per cc.) was used in place of the suspension. In the latter cases the mixtures were usually filtered immediately; in the former after standing for several days. Similar results were obtained at room temperature and at 37·5°.

Table II.

	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$		
	Phosphate solution	Ringer solution	Blood-serum
Lead suspension	0·62	1·0	1·1
Lead acetate	0·62	0·69	1·1

These values are each the average of several experiments[1] (3-7). The addition of small volumes of lead suspension to Ringer solution or serum does not change the p_H appreciably (the p_H of the mixture being calculated from the bicarbonate concentration of the ultra-filtrate)[2]. The decrease in bicarbonate concentration due to dilution by the suspension is balanced by the bicarbonate formed in the reaction, *e.g.*

$$Pb(OH)_2 + HPO_4'' + 2CO_2 \rightarrow PbHPO_4 + 2HCO_3'.$$

The ratios in Table II fall into two classes which correspond approximately with the ratios for the secondary phosphate $PbHPO_4$ and the tertiary phosphate $Pb_3(PO_4)_2$, *i.e.* 1·0 and 0·67 respectively. Assuming that the results represent the formation of these compounds, it follows that the secondary phosphate is formed when lead acetate or lead suspension is added to serum, whilst with Ringer solution lead suspension gives the secondary compound and lead acetate the tertiary. The tertiary phosphate is formed when either the suspension or lead acetate is added to aqueous phosphate solution. These differences can hardly be due to the effect of p_H since with the phosphate solution a ratio 0·62 was obtained both with lead acetate (p_H of mixture *ca.* 6·0) and lead suspension (p_H of mixture *ca.* 8·5), whereas with Ringer solution or serum (p_H of mixture *ca.* 7·5)[3] the two ratios, 1·0 and 0·67, were obtained. It seems preferable to attribute the formation of the secondary phosphate under certain conditions to delayed equilibrium[1] even though the same ratios as those quoted above were obtained after the mixtures had stood for 14 days. It may be noted that the greater the complexity of the system the greater is the tendency to give the secondary phosphate.

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[1] The values of a given series (*e.g.* Table I) agreed well between themselves. Greater deviations (5 %) were obtained between the different series. This may have been due to the slight differences in tint sometimes obtained between the standard and the experimental solutions in the colorimetric method.

[2] With the mixture in Table IV, $[NaHCO_3]$ of mixture ultra-filtrate $= 0·0266$.

[3] Similar results were obtained with Ringer solution and serum in the absence of the gas mixture (p_H of mixture $> 8·5$).

Rate of combination of phosphate ions with the lead suspension.

The rate of combination was measured by filtering portions of the mixture at various time intervals and estimating the uncombined phosphate in 2 cc. of filtrate. The amount of suspension used was such as to combine with approximately two-thirds of the phosphate. Under the conditions employed the preliminary oxidation of metallic lead to lead hydroxide was complete before filtration was commenced.

Two series of experiments were carried out with Ringer solution and serum at 20° and 37·5°. The filter (with the exception of the manometer) was immersed in a thermostat. The results are given in Tables III and IV. The times are reckoned from the time of mixing to the mid-time of filtration.

Table III.

75 cc. Ringer solution.
4·5 cc. lead suspension = 20·7 mg. total Pb.

	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$	
Time (hours)	20°	37·5°
0·5	0·92	0·96
1·5	0·93	0·96
3·0	0·95	0·98
5·0	0·95	0·97
8·3	0·98	0·98

Table IV.

50 cc serum (0·00196 *M* phosphate).
3·0 cc. lead suspension = 13·8 mg. total Pb.

	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$	
Time (hours)	20°	37·5°
2·0	0·83	0·95
6·0	0·86	0·98
25·0	0·95	0 99
50·0	0·97	1 04
73·0	1·04	1 00

When these values are plotted (cf. Fig. 2) the curves show a rapid rise in the amount of combined phosphate up to approximately 90 % of the final amount, followed abruptly by a much slower combination of the particles with the remaining 10 % of phosphate. The greater portion of the reaction is too rapid to be investigated by the present method. It can be seen, however, that at 20° the rate of combination in serum is somewhat slower than in Ringer solution. At 37·5°, although the rates are similar, the degree of dispersion of the suspension in the two liquids is very different (see below).

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[1] Holt, La Mer and Chown [1925], who have studied the solubility of the calcium phosphates in water, found under certain conditions where the tertiary salt was the least soluble that the secondary phosphate was initially formed and that the subsequent transformation into $Ca_3(PO_4)_2$ was slow.

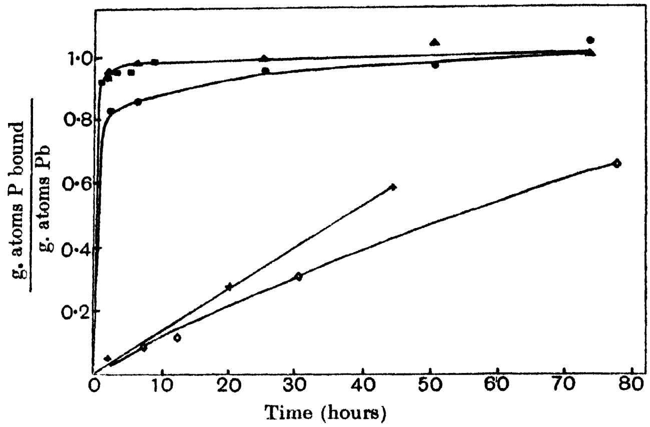

Fig. 2.

▲ Serum and lead suspension at 37·5°.
■ Ringer solution and lead suspension at 20°.
● Serum and lead suspension at 20°.
◇ + Serum and lead carbonate suspension at room temperature.

The rates in serum and Ringer solution were compared under more comparable conditions by preparing a Ringer solution containing the same concentrations of bicarbonate and of phosphate as were contained in the serum. These were 0·0312 and 0·00204 *M* respectively. The results are given in Table V.

Table V.

Room temperature.
50 cc. serum or Ringer solution.
3 cc. lead suspension = 16·2 mg. total Pb.

	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$	
Time (hours)	Ringer	Serum
1·5	0·92	0·80
7·0	0·92	0·83
8·0	0·93	0·86
26·0	0·96	0·90

The slower rate in serum is probably due to the presence of a film of serum proteins round the particles of the suspension.

It was thought that the rapid decrease in the rate of combination might be accounted for by the decrease in the area of reacting surface with time and also possibly to a decreasing rate of penetration of the phosphate ions through the layer of lead phosphate. An attempt was made to test these points using a series of results obtained with the ultra-filter used by Giemsa[1] [1922]. It was assumed that the velocity of chemical reaction at the surface was rapid compared with the rate of diffusion of phosphate ion. The calculated values of the velocity constant were found to fall rapidly with time. These values suggested that the process, instead of being governed throughout by the rate of diffusion, consisted of a reaction accompanied and eventually superseded by a much slower reaction, which was therefore presumably slow compared to the rate of diffusion of phosphate ion. The following facts support the view that the rapid reaction is a combination of phosphate and lead hydroxide and the slow reaction the combination of phosphate and lead carbonate.

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[1] The rate of filtration with this type of ultra-filter was high using aqueous phosphate or Ringer solution.

(1) In the rapid reaction 90 % approximately of the combining phosphate disappears from solution. Approximately 10 % of the lead is actually present at the beginning as carbonate.

(2) It was found that the reaction proceeded at the same rate whether the mixture was vigorously stirred or maintained at rest. (The method could probably not detect any influence of stirring on the rapid reaction if such were present.)

(3) The very small solubility of lead carbonate in Ringer solution and serum (Jowett).

To obtain further information regarding the behaviour of lead carbonate with regard to phosphate ion the rate of combination of a coarse suspension of lead carbonate[1] with the phosphate ions of serum was measured. The results are given in Table VI and Fig. 2. The difficulty of obtaining a uniform sample of the suspension makes the absolute values in column 2 somewhat uncertain. The particles of the suspension used in (A) were smaller than those in (B); this probably accounts for the faster rate in the former case.

Table VI.

Room temperature.

(A) 50 cc serum (0·00203 *M* phosphate).
3·0 cc. lead carbonate suspension = 1·2 % Pb.

Time (hours)	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$	k
2	0·04	0·038
20	0·27	0·028
44	0·58	0·032

(B) 50 cc. serum (0·00203 *M* phosphate).
3·0 cc. lead carbonate suspension = 1·08 % Pb.

Time (hours)	$\frac{\text{g. atoms P bound}}{\text{g. atoms Pb}}$	k
7	0·08	0·021
12	0·11	0·017
30	0·30	0·020
77	0·65	0·021

In both cases excess lead carbonate was present which combined finally with all the phosphate present. In spite of the decrease in phosphate concentration the velocity remains practically constant. Assuming that the velocity is proportional only to the reacting surface (*i.e.* assuming that the chemical change is slow compared with the rate of arrival of phosphate ions at the surface) and that $PbHPO_4$ is formed, we would write

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[1] Prepared by mixing solutions of lead acetate and sodium bicarbonate both containing 0·5 % gelatin. The suspension settled almost completely in 60 minutes.

$$\frac{dx}{dt} = k\,(a-x)^{\frac{2}{3}},$$

a = initial g. mol. $PbCO_3$, x = g. mol. phosphate disappeared in time t, whence

$$k = \frac{1}{t}\,[a^{\frac{1}{3}} - (a-x)^{\frac{1}{3}}].$$

The calculated values of k are given in the third column, and are reasonably constant. The velocity of combination is also not inconsistent with the assumption that in the case of the experiments in Tables III–V the later slow disappearance of phosphate is due to the lead carbonate present in the lead suspension.

The lead ion content.

No detectable amount of ionic lead was found in the small volume of ultrafiltrates from the mixtures of serum (or Ringer solution) and the lead suspension. This would be expected, for however slowly the lead particles were converted into phosphate the lead ion concentration in the bulk of the serum would be governed by the solubility product of the phosphate and hence would be very small.

It may be noted that the values sometimes given for the so-called solubility of metallic lead in serum bear no relation to the resulting lead ion concentration. Such a solubility would depend chiefly on the amount of oxygen available for the oxidation of the lead to lead hydroxide and the peptising action of the serum on this compound or on lead phosphate formed therefrom.

The physical state of the lead particles in serum, etc.

The lead suspension was added to water, the phosphate solution, Ringer solution, and serum and the rate of settling at room temperature and at 37·5° observed. The rates were in the following order:

Ringer solution > water, serum > phosphate solution.

With the exception of Ringer solution at 37·5° (where precipitation occurs within 5 minutes) and to a lesser degree at room temperature, there is no marked acceleration of the slow rate of settling which takes place in the lead suspension itself.

The particles of the lead suspension are negatively charged. The increased stability in the phosphate solution may be due to an increase in this charge on conversion into lead phosphate and the decreased stability in Ringer solution may be due to the presence of the calcium ion. In serum there is the protective effect of the proteins to counterbalance any precipitating action of the various cations present. It may be noted in this connection that the addition of small amounts of lead acetate solution to serum gives a finely dispersed sol of lead phosphate [cf. Fairhall *et al.*, 1926, p. 24].

CONCLUSIONS.

It can be concluded from the results obtained that when the lead suspension is injected into the blood-stream the transformation of lead to lead hydroxide, and hydroxide to phosphate is rapid if there is rapid mixing with a sufficiently large volume of blood. In view of the speed of distribution of a dye-stuff throughout the blood (in about 5 minutes; cf. Lindhard [1926]) it is not improbable that a sufficient degree of mixing is attained to ensure the maximum velocity of chemical changes. Consequently the concentration of ionic lead in serum will fall almost immediately to that corresponding to the solubility of secondary lead phosphate. If, however, the particles temporarily reduced the phosphate concentration in the admixed serum to a very low value[1] the formation of lead carbonate would take place owing to the large amounts of bicarbonate ions present. Further mixing with more blood would again reduce the lead ion concentration to the value characteristic of lead phosphate.

Since, however, any given amount of a soluble lead salt under similar conditions would be transformed into lead phosphate (or carbonate) more rapidly than an equal amount of the lead suspension itself, it is necessary to explain the greater toxic effect of injection of the simple lead salt. It might be suggested that with the soluble salt a faster temporary local reduction of phosphate and bicarbonate concentration to a very low value would leave enough ionic lead in circulation for a sufficient time to cause injury. With the lead suspension the concentration of ionic lead could never be greater than that corresponding to lead hydroxide. An alternative and more probable view is that the fine sol of lead phosphate which would be formed from the soluble lead salt would be more toxic (greater number of smaller particles facilitating distribution, penetration, etc.) than the coarser suspension resulting from the introduction of the lead in the form of the lead suspension.

SUMMARY.

1. The changes which take place when a colloidal lead suspension is added to an aqueous solution of sodium phosphate, to a Ringer solution containing sodium bicarbonate and phosphate, and to cow's serum have been investigated.

2. Ionic lead is not produced in any detectable amount on the addition of the lead suspension to these solutions. The phosphate ions unite with the particles which remain in suspension (except in the case of Ringer solution at 37·5°).

3. The final values obtained for the ratio g. atoms phosphorus bound/g. atoms lead present correspond with the formation of the tertiary phosphate $Pb_3(PO_4)_2$ in the aqueous phosphate solution. On the other hand, the secondary

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[1] Assuming the plasma volume to be 1·515 l. [Lindhard, 1926] and an inorganic phosphorus content of 3·72 mg. per 100 cc. there is sufficient phosphate present to combine with 0·38 g. Pb as $PbHPO_4$. The maximum amount of lead suspension injected is 20 cc., i.e. 0·1 g.Pb.

phosphate $PbHPO_4$ is formed in Ringer solution and in serum. Using lead acetate in place of the suspension, the secondary phosphate is formed in serum and the tertiary salt in the other two cases. The formation of the secondary phosphate is attributed to delayed equilibrium.

4. The combination of phosphate ions with the particles of the lead suspension is preceded by the rapid oxidation of the metallic lead to lead hydroxide. The disappearance of the phosphate is due to two simultaneous reactions: (*a*) a rapid combination with the lead hydroxide, (*b*) a slow combination with the lead carbonate of the suspension. The results obtained with a suspension of lead carbonate alone show that the velocity of the latter reaction is not governed by the rate of diffusion of the phosphate ions to the reaction surface of the particles but by the slow chemical reaction at the surface.

In conclusion, the writer wishes to express his best thanks to Prof. W. C. M. Lewis, F.R.S., under whose direction this work was carried out.

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CIV. ANTINEURITIC YEAST CONCENTRATES.

II. THE USE OF NORITE CHARCOAL IN THE CONCENTRATION OF TORULIN.

By HENRY WULFF KINNERSLEY
AND RUDOLPH ALBERT PETERS.

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(*Received June 2nd, 1927.*)

In this paper are described improved methods of concentrating torulin to an activity of 0·5–1·0 mg. per day and some of the experimental evidence upon which the better methods are based. *Torulin* is here defined as the factor in yeast curative of symptoms in pigeons of head retraction induced by feeding upon polished rice. The work forms a continuation of that previously published [Kinnersley and Peters, 1925]. Discussion of the relation of this work to that of others is postponed to the end of this paper.

EXPERIMENTAL.

The methods of testing have been those previously described, and the activity (curative and protective) is given throughout in mg. per pigeon per day (day-dose). Previous methods of concentrating to an activity of 1·0 mg. involved a preparation of the Osborne-Wakeman aqueous extract from baker's yeast, and the successive removal of precipitates with neutral lead acetate and acid mercuric sulphate. At this stage in the earlier experiments, barium sulphide together with barium sulphate precipitated in the solution was used to clear metals and certain colloidal matter before charcoal treatment. Subsequently this was discarded owing to the tedium of filtration. Instead, the filtrate from the mercuric sulphate precipitate was brought to p_H 5·0 approx., any precipitate which appeared being removed. Charcoal was then stirred into the solution, and the antineuritic substance together with impurities removed from the charcoal by extraction with acid 50 % alcohol. The idea which guided these experiments was based upon the belief that the earliest possible treatment with charcoal shortened stages in which large volumes of solution must be handled. Working upon the small scale and with care in the use of the mercury reagent, satisfactory concentrates can be prepared in this way. The concentrates so obtained can be further purified by alcohol fractionation. Exp. 1 gives the details of one such experiment and exemplifies the technique.

Exp. 1. 7 lb. baker's yeast (D.C.L.) were extracted twice with tap water, the combined extracts making a volume of 2700 cc. and containing 100·9 g. organic solids. 280 cc. neutral lead acetate (25 %) were added, and to the filtrate sulphuric acid until acid to Congo red and then 70 cc. of mercuric sulphate reagent (Hopkins). The filtrate was brought to p_H 5·0 with NaOH (20 %), and a small precipitate removed by filtration. It was treated successively with 30 and then 10 g. norite charcoal, allowed to stand with stirring for about 10 minutes, and the charcoal collected upon a filter and well washed with distilled water. The combined charcoals were extracted with 200 cc. and 100 cc. 55 % alcohol by volume containing 1 cc. conc. HCl %. The organic solids present in the combined alcoholic extract were 2·850 g. The solutions were concentrated to about 5 cc. *in vacuo* at about 60°, and 45 cc. 97 % alcohol were added, making an alcohol concentration of 87 %[1]. The precipitate was removed by the centrifuge and washed with 87 % alcohol. The further treatment is sketched below:

Centrifugate Test 500 + 700, 700 doses Alcohol removed, 50 cc. water added, and lead acetate (25 %) until no further precipate	87 % alcohol precipitate
Centrifugate. Passed H_2S 2 hours and washed precipitate with water containing trace of acetic acid	PbAc precipitate
Centrifugate. Boiled off H_2S and concentrated *in vacuo* to 4 cc.; added 95 cc. alcohol	Sulphide precipitate
Centrifugate Test 600 doses Concentrated with 1 cc. conc. HCl (to remove acetic) to dark brown gum. Extracted with hot 97 % alcohol. Washed the precipitate with 97 % alcohol	95 % alcohol precipitate
Centrifugate. Removed alcohol and added NaOH (20 %) to p_H 5·0. To the volume 10 cc. added 90 cc. 97 % alcohol	97 % alcohol precipitate
Centrifugate. Concentrated to about 2 cc. and added 48 cc. 97 % alcohol. The precipitate was redissolved in 3 cc. H_2O and reprecipitated with 47 cc. 97 % alcohol	88 % alcohol precipitate
Centrifugate Test 330 doses Concentrated nearly to dryness, added 45 cc. absolute[2] alcohol, washed the precipitate with 97 % alcohol	92 % alcohol precipitate
Centrifugate. Concentrated to dark gum, extracted with hot absolute alcohol, the precipitate being washed with absolute alcohol	97 % alcohol precipitate
Centrifugate. Taken to dryness, and extracted with 50 cc. ho absolute alcohol Test 700 doses. Organic solids 0·966 g.	98/99 % alcohol precipitate

This experiment is chosen out of many, because though the yield was not so good as in others or the final activity quite 1 mg., it illustrates certain points, in particular the best method of fractionating with alcohol. It appears to represent the simplest possible method of reaching this activity from the charcoal concentrates previously described. The principle involved is the removal of some interfering substance with lead acetate, removal of metals in acetic acid solution, followed by gradual removal of acetic acid and material insoluble in alcohol in acid solution. This is followed by a repetition of the same steps in rather more alkaline solution. The steps are fairly expeditious. Experience has convinced us that it is dangerous to venture from the path described. For instance, too rapid use of alcohol does not take to pieces the adsorption complexes efficiently, and there is much loss of activity upon the alcohol precipitates. Omission of the HCl after treatment with H_2S leads to the throwing out of torulin upon the alcohol precipitates. Improper removal of alcohol before bringing to p_H 5·0 is apt to cause destruction when soda is used, etc. It is abundantly clear that torulin of this activity is soluble in absolute ethyl alcohol containing mere traces of water.

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[1] By volume. Concentrations of alcohol are to be taken throughout as concentration by volume.

[2] No precautions to dry the absolute alcohol have been taken.

The method exemplified in Exp. 1 can be relied upon to give some 700–800 doses from 7 lb. D.C.L. baker's yeast of an activity of about 5·0 mg. a day at the charcoal stage and about 1 mg. per day after alcohol fractionation. A trial of brewer's yeast did not give more activity, and the material was much more coloured.

When these methods were applied to large scale batches of yeast (56 lb.), it became evident that other factors intervened, complicating the subsequent fractionation. These and the slow filtration of the earlier stages led to a return to a more careful study of the pre-charcoal steps. It was found that by the removal of gum and subsequent adsorption of the torulin upon norite charcoal at p_H 7·0, the yield of active substance may be doubled, and the activity at the charcoal stage improved to between 0·5–1·5 mg. per day. Moreover, the speed of preparation is so much increased that it is possible for two persons to work up 14–28 lb. yeast in 2–3 days to give a yield of 3000–6000 day-doses of this activity.

It is proposed to present certain of the experimental evidence bearing upon the method of extraction used.

(1) *The use of baryta to clear gum and other colloidal matter.*

It has been found that treatment with cold saturated baryta after removal of the neutral lead acetate precipitates under certain circumstances rapidly flocculates the gum and clears the solution. Immediately following this step, sufficient sulphuric acid can be added to make acid and throw down the barium as sulphate which rapidly settles. This treatment much accelerates filtration in the subsequent stages. Certainty as to the exact mechanism of clearance has not yet been reached. It appears to be essentially a colloidal flocculation. It is suggested as an explanation that in the presence of traces of lead, baryta removes the gum as a coarse flocculent precipitate which in its turn adsorbs other colloidal substances. It is certainly not simple because the optimum conditions vary slightly according to the condition of the yeast. Provided that the amount of lead is kept low, the losses at this stage are small. This is not so if the solution is cleared by the addition of baryta and basic lead acetate. In one case in which this was tried, a magnificent clearance was effected, but at the expense of large losses of active material by adsorption upon the precipitate. Table I gives some results.

Table I.

Exp.	Condition of material	Dose given	Bird test (days)	Total doses
2	Pb filtrate before baryta	1/3000	4·0	12,000
	Same after baryta	1/2200	5·0	11,000
3	Aqueous yeast extract before lead acetate	1/1000	3·5	3,500
	Same after lead and baryta stage	1/1000	6·0	6,000

In Exp. 3 there is an apparent increase in activity. As this has been noticed more than once it is probably not due to experimental error and is analogous to the increase in growth-promoting activity for yeast observed by Funk and Dubin [1920] after treatment of yeast extracts with lead acetate and mercury. It suggests that any estimates of actual torulin content obtained by the feeding of a natural food-stuff are apt to be erroneous. The inhibitory effect of impurities may be due either to an actual toxic antagonism or merely to a failure of absorption in the gut.

(2) *Specificity of adsorption by charcoal.*

Charcoal as an adsorbent for the antineuritic factor from rice polishings was first employed by Chamberlain and Vedder [1911, 1912]. These authors were not able to recover the active principle from the charcoal by extraction with ether, absolute alcohol or water. Cooper [1913] confirmed the extraction by animal charcoal from horse-flesh extract, but stated that water and alcohol would recover the factor. Eddy, Heft, Stevenson and Johnson [1921] used charcoal (norite) for the adsorption from alfalfa extract of the growth-promoting substance for yeast, part of which they attributed to vitamin B. They recovered the principle with glacial acetic acid. Funk and Dubin [1921] have employed norite for fractionating vitamin B and the yeast growth-promoting factor. Mueller [1922] used it for separating streptococcal growth-factors.

Earlier in this work the active principle was recovered from norite charcoal by the use of 50 % alcohol by volume containing 1 cc. HCl %. More lately this treatment has been varied, and also the norite charcoal itself purified as described by Merrill [1921] by boiling with strong HCl, washing until free from chlorides and drying. Such purified norite does not seem to be changed in adsorptive properties so far as this work is concerned. In the attempt to improve the use of norite for concentrating torulin, it became of importance to settle how far adsorption upon charcoal was a property of torulin itself. After treatment with mercuric sulphate, adsorption at p_H 5·0–7·0 is a property of the torulin in solution. Exp. 4 shows that it is not necessarily a property of torulin at a more active stage, viz. 1·0 mg. day-dose.

Exp. 4. Preparation from baker's yeast, made by the older technique and worked up to solubility in 99 % alcohol. Organic solids 0·525 g. Bird tests: 7 Aug. 1/100, 5 days; 26 Oct. 1/100, 4 days; activity 1·2 mg. Alcohol removed *in vacuo*, taken up in 50 cc. H_2O and brought to p_H 5·0 with NaOH, a small insoluble precipitate being removed by the centrifuge. Treated with 1 g. of washed norite charcoal, which largely decolorised the preparation. Filtrate from charcoal gave organic solids 0·425 g. Bird tests: 1/50, 8 days; 8 days, or 100 doses; activity 1·05 mg.

Hence at p_H 5·5 at a later stage charcoal does not extract torulin from solution when 10 doses to the cc. are present, although at an earlier stage it extracts it when not more than one dose is present to 1 cc. It is still possible to obtain some adsorption of torulin at p_H 7·0 and thereby a concentration of the active principle from a solution of activity 1·0 mg., provided that the torulin concentration is 50–200 doses per 10 cc., but a second treatment of such a solution adsorbs no more torulin, so that even here torulin is not itself being adsorbed.

It may be concluded therefore that adsorption of torulin by norite charcoal is not a property of torulin itself, but that it depends upon the presence of some co-adsorbent. The problem in the early stages therefore narrows down to the issue of removing the maximum of interfering substances without removing the co-adsorbent.

(3) *The optimum conditions for adsorption.*

This conclusion leads to the following three questions. What is the optimum p_H for adsorption? Does the optimum p_H differ for material that has undergone previous treatment with lead and baryta only, as distinct from that which has also been treated with mercuric sulphate? Is treatment with mercuric sulphate necessary? Exps. 5–9 were designed to settle these questions, all except the last being performed upon an extract from 56 lb. of baker's yeast, kindly made by Messrs The British Drug Houses, Ltd., so that the basic extract should be homogeneous. In Exp. 5 adsorptions at various p_H were made after mercuric sulphate treatment. In Exp. 6 the effect of a re-extraction at p_H 5·0 after one at p_H 1·0 was tried. In Exp. 7 adsorption at various p_H in absence of mercuric sulphate was tried, this being reinvestigated in Exp. 8.

Exp. 5. Preliminary treatment—lead acetate, baryta, and mercuric sulphate. The total filtrate from these precipitates, equivalent to some 2800 day-doses, was divided into four equal portions, which were brought by addition of NaOH to p_H about 1·0, 4·0, 6·0 and 7·0. Each portion was then treated with 15 g. followed by 5 g. of washed norite, each lot of charcoal being then treated with acid 50 % alcohol. Table II shows the tests for the 15 g. charcoal extracts.

Second charcoal extracts in the case of p_H 6·0 and 7·0 yielded 180 and 72 doses of activity 3·4 mg. and 9 mg.

Table II.

Exp.	p_H	Organic solids (g.)	Pigeon tests (doses)	Activity (mg.)	% extraction
5	1·0 approx.	1·248	186	6·7	27
	4·0	0·990	$\left.\begin{matrix}164\\82\end{matrix}\right\}$ 120	8·2	17
	6·0	1·064	$\left.\begin{matrix}456\\760\end{matrix}\right\}$ 603	1·8	86
	7·0	1·275	$\left.\begin{matrix}850\\680\end{matrix}\right\}$ 760	1·7	108
6	5·0 (after 1·0)	0·976	$\left.\begin{matrix}120\\270\end{matrix}\right\}$ 200	4·8	—

The most efficient extraction was at p_H 7·0, where in this case within the limits of error practically all the torulin was removed by one charcoal treatment, to yield material of an activity below 2 mg. The result at p_H 6·0 is near to this. The experiment shows how profoundly the charcoal adsorption is influenced by p_H.

We may next consider the effect of treating the filtrate from p_H 1·0 with fresh charcoal at p_H 5·0. This is seen in Exp. 6 (Table II). Assuming that some 100 doses were extracted by the second charcoal treatment at p_H 1·0 (this test was accidentally omitted), which is certainly an outside figure, there should be at least 414 doses remaining. Of these not more than half can be extracted at p_H 5·0 following the first charcoal treatments. It will be noticed that irrespective of p_H about 1·0 g. of organic solids is adsorbed by 15 g. of charcoal. After adsorption 48 g. of organic solids remained in the filtrate.

It may be asked now what is the effect of omitting mercuric sulphate, a step that is undesirable from several points of view. Exp. 7 (Table III) deals with this point.

Exp. 7. A volume of solution which had not been treated with mercuric sulphate, equivalent to some 2100 doses, was divided into three portions and treated as before with 15 g. washed norite charcoal, after being brought respectively to p_H 5·0, 6·0 and 7·0 with NaOH.

Table III. (*Exp.* 7.)

p_H	Organic solids (g.)	Pigeon test (doses)	Activity (mg.)	% extraction
5·0	1·300		3·4	55
6·0	1·036	$\left.\begin{matrix}200\\400\end{matrix}\right\}$ 300 av.	3·4	43
7·0	1·170	304	3·9	43

In this experiment adsorption has been relatively inefficient, The presence of the substances in solution precipitable by mercuric sulphate has therefore depressed the adsorption at p_H 6·0 and 7·0 as compared with Exp. 5. This result was curious but has received later confirmation. In Exp. 8 the remainder of the batch was treated at p_H 6·0 and 7·0.

Exp. 8. The remaining material from the last experiments, as in Exp. 7, was treated at p_H 6·0 and 7·0 with 60 and then 20 g. of washed norite charcoal.

The method of extraction was varied in this case. Successive extractions with $N/10$ HCl, and then 50 % acid alcohol were employed for the first charcoal, and with 50 % acid alcohol alone for the second. Table IV gives the results.

Table IV. (*Exp.* 8.)

p_H	Charcoal (g.)	Extract by	Organic solids (g.)	Doses	Activity (mg.)	% extraction
6·0	60	$N/10$ HCl	1·127	840 749 } 1589	1·35	—
		50 % alc.	1·926		2·6	50
	20	50 % alc.	1·430	440	3·2	14
7·0	60	$N/10$ HCl	1·044	725 440 } 1165	1·44	—
		50 % alc.	2·354		5·4	38
	20	50 % alc.	1·44	?840*	—	—

* Probably an irregular bird.

Exp. 9 is a further variation of the same type. The extract from 14 lb. baker's yeast after treatment with lead acetate and baryta, and removal of baryta with sulphuric acid yielded a filtrate of 10,000 cc., which was divided into two equal portions *A* and *B*. To *A* 50 cc. mercuric sulphate reagent were added and the whole filtered immediately. To the filtrate from *A* and to *B* (untreated with mercury), sufficient NaOH was added to bring the p_H to 7·0. Each was then treated successively with 30 and 10 g. washed norite. Subsequently the charcoals were extracted with $N/10$ HCl and 50 % acid alcohol. Table V gives the results for the first charcoal (30 g.) extracts.

Table V. (*Exp.* 9.)

	Extract by	Organic solids (g.)	Doses	Activity (mg.)
A.	$N/10$ HCl	0·440	900 900+	0·49
	50 % alc.	1·400	1800+	0·78
B.	$N/10$ HCl	0·560	600+	0·93
	50 % alc.	1·592	Nil 240	6·6 ?

Exps. 8 and 9 seem to confirm the fact that treatment with mercuric sulphate facilitates adsorption of torulin. It is possible to remove the substance precipitated by mercury in large part by treatment with charcoal at p_H 2·0, and to follow this with the usual adsorption at p_H 7·0. Experiments upon the yield of material show that it is as good for the $N/10$ HCl extract both as regards amount and activity, but the yield extracted by 50 % acid alcohol is not so good as following the mercury treatment. A detailed account of this method of treatment is given later, as it is likely to prove of value to those who require these concentrates for feeding experiments, owing to the complete freedom from mercury. Enough evidence has been adduced to show how variable is the character of an adsorption of this kind, and how dangerous it might be to draw conclusions about the adsorption of torulin from one class of mixture such as yeast extract and apply this to another. Such experience may be regarded as familiar to the enzyme investigator, as shown by the work of Willstätter and his colleagues upon invertase.

The experiments in this section permit us to draw the following conclusions in answer to the questions. The optimum p_H varies according to the mixture from which adsorption takes place. For material not treated with mercuric sulphate the optimum is p_H 5·0–6·0, and this adsorption is less efficient than adsorption at p_H 7·0 after treatment with mercuric sulphate. The mercuric sulphate stage or its equivalent cannot be omitted if the maximum yield is to be obtained.

(4) *The optimum conditions for extraction.*

It will have been observed in Exps. 8 and 9 that two extractions of the charcoal were made, namely, with *N*/10 HCl followed by 50 % acid alcohol. Further, the material extracted by *N*/10 HCl in this way tends to have a high activity, 0·4 mg. having been observed at this stage. In Exp. 9 the activity was 0·5 mg. This was rather unexpected in view of earlier experiments and suggested that the gum had modified conditions in previous work. It further appeared possible that the use of stronger alcohol as a first step might produce a differential extraction, and give even higher activity in the extract. Exp. 10 illustrates the effect of extracting first with strong alcohol.

Exp. 10. 14 lb. baker's yeast. Extraction of the charcoal was carried out first with 300 cc. 97 % alcohol containing 1 cc. conc. HCl %; allowing for water retained by the charcoal this means extraction with 85–90 % alcohol by volume. Subsequent extractions were with *N*/10 HCl and 60 % acid alcohol. Table VI gives the results obtained for the first and second charcoals.

Table VI.

Extract by	Organic solids (g.)	Doses	Activity (mg.)
1st charcoal:			
acid 97 % alcohol	2·450	1250 1250	2·0
N/10 HCl	0·560	500 150	1·7
acid 60 % alcohol	1·080	300 300	3·5
2nd charcoal:			
acid 97 % alcohol	0·852	360 360	2·5
N/10 HCl	0·130	100 200	0·9
acid 60 % alcohol	0·231	under 50	? 4·5

Total doses 1875 + 560 = 2435.

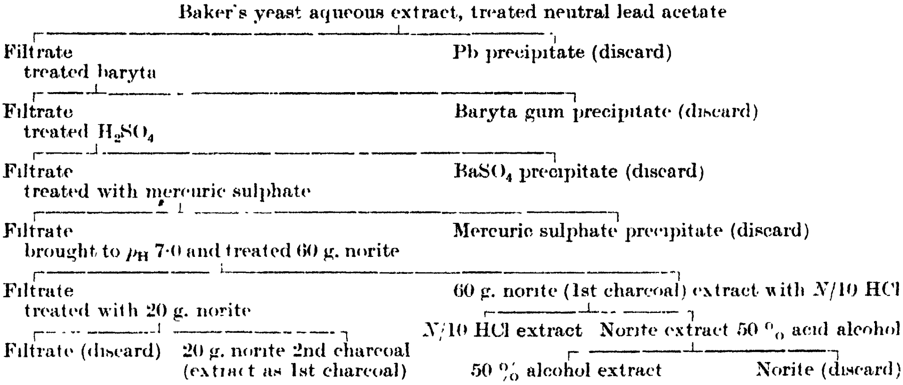
The material obtained in this way is not so good as that from the initial *N*/10 extraction. Nothing is therefore to be gained by the insertion of the strong alcohol step. It is clearly ineffective as a means of removing impurities, owing to the large number of doses extracted by the strong alcohol.

It may be further enquired whether anything is to be gained by an extraction with stronger alcohol following 50 %. This was tried in one case giving no appreciable yield of torulin although organic substances were still extracted from the charcoal. In view of the above results, as a routine, two extractions of the charcoal are now made, one with $N/10$ HCl to which sufficient HCl is added to make the p_H definitely acid to Congo red if necessary. A further extraction is then made with 50 % acid alcohol by volume.

Optimum amount of charcoal. Experiment has shown that for the aqueous extract from 14 lb. of yeast, treatment with 60 g. of charcoal followed by a subsequent 20 g. cannot be improved.

Improved technique for concentrating torulin.

Based upon the foregoing experiments, the following technique has been elaborated.


Baker's yeast aqueous extract, treated neutral lead acetate
Filtrate treated baryta
Pb precipitate (discard)
Filtrate treated H_2SO_4
Baryta gum precipitate (discard)
Filtrate treated with mercuric sulphate
$BaSO_4$ precipitate (discard)
Filtrate brought to p_H 7·0 and treated 60 g. norite
Mercuric sulphate precipitate (discard)
Filtrate treated with 20 g. norite
60 g. norite (1st charcoal) extract with $N/10$ HCl
Filtrate (discard)
20 g. norite 2nd charcoal (extract as 1st charcoal)
$N/10$ HCl extract
Norite extract 50 % acid alcohol
50 % alcohol extract
Norite (discard)

Preparation of norite concentrate.

The description applies to 14 lb. of baker's yeast (D.C.L.). The yeast is allowed to remain 3 days at laboratory temperature, 15–20°, in the 7 lb. bags. Less yield seems to be obtained if this preliminary autolysis is omitted. An aqueous extract is then made by stirring the yeast gradually in small quantities into 3500 cc. of boiling tap water until all the yeast is added. (We cannot find any difference in results if acetic acid be added.) The mixture is not allowed to boil for more than 5 minutes. It is then filtered upon Büchner funnels with constant renewal of papers. This process takes about 2 hours. A second extraction with 2800 cc. of water is made. The whole operation of extraction takes about 4 hours and gives a volume of 7000 cc. The combined filtrates are treated with about 400 cc. of 25 % neutral lead acetate solution. This amount is more than the amount necessary to bring down the main bulky precipitate, but is short of complete precipitation. It seems to be the optimum for the next stage. The lead precipitate is removed by filtration overnight through folded filter papers in ordinary funnels. The speed of this filtration varies, most preparations filtering in a few hours. No attempt is made to wash the precipitates. The filtrate often clouds upon standing, but need not be filtered again on this account. The filtrate from the lead precipitate measuring some 5500 cc. is treated with cold saturated baryta solution containing a little precipitated baryta in suspension. About 3000–4000 cc. are usually required. Immediate flocculation of the gum should take place which should then settle rapidly to the bottom of the vessel, clearing the solution to leave a crystal clear lemon yellow supernatant fluid.

It is well to test this step upon a small scale (50–100 cc.). If flocculation is not obtained by the addition of baryta, a small amount of the lead acetate solution, 2 cc. per litre, can be added, *before* adding the baryta, which will usually settle matters (see Section (1) of this paper). After flocculation of the gum, filtration is rapidly effected through folded papers, and the filtrate treated as soon as possible with sufficient sulphuric acid to make acid to Congo red. The barium sulphate, etc., are removed by decantation and filtration. At this stage the volume of the solution may measure about 10 litres.

Occasionally we have met with a yeast extract which will not filter overnight at the lead acetate stage. If this be so, the only course is to treat directly with baryta in the presence of the lead precipitate. The yield from such a preparation is not so good, the precipitates adsorbing torulin.

The filtrate from the barium sulphate, which need not be absolutely clear, is brought to p_H 2·5 (approx.) with addition of either sulphuric acid or of NaOH, and is then treated with mercuric sulphate in sulphuric acid, 80–100 cc. being used. It is then filtered through folded papers without delay. After filtration a faint cloud often appears in the solution upon standing, which is not removed. Care must be taken not to have excess of mercury present at this stage. The point to which we work is reached when 10 cc. of the solution taken into a test-tube ceases to give an immediate flocculent precipitate with addition of one drop of the reagent. At this point it gives a cloud.

After removal of the mercury precipitate, NaOH is added to p_H 7·0 (at which point the faint cloud if present redissolves) and the solution is treated with 60 g. dry purified norite charcoal. The charcoal is allowed to stand in contact with the solution with stirring for about 10 minutes, and is then collected upon a Büchner funnel. Filtration is usually rapid at this stage. The filtrate from the first charcoal is treated with 20 g. more of charcoal, this second charcoal being kept separate from the first. The charcoal precipitates are thoroughly washed with distilled water upon the funnel. The first and second charcoals are each treated separately as follows. Two extractions are made with $N/10$ HCl, *ca.* 200 cc., upon the hot water-bath, the papers and charcoal being placed with the HCl for this purpose in a beaker. If the extracting solution during the boiling becomes alkaline to Congo red, strong HCl is added drop by drop until the whole is acid to Congo red paper. After heating for 1 hour, the charcoal is filtered off while hot. A second extraction of the charcoal is then made in the same manner. These two almost colourless extracts are combined, dilute $BaCl_2$ is added to remove the last traces of sulphuric acid and the extracts are concentrated *in vacuo* at a temperature not exceeding 60° to a volume of 50 cc. If a cold store is not available, sufficient alcohol is added to make a volume concentration of about 15 %. This acts as a preservative; in its absence we have found moulds growing in such solutions.

After extraction of the charcoal with $N/10$ HCl, a further yield of less active material can be obtained by extraction with 50 % alcohol (by volume) containing 1 cc. strong HCl %; two extractions are made, each with about 150 cc. acid alcohol.

The second charcoal extracts are worked up similarly; lately the extraction with 50 % acid alcohol has been omitted. The above operations take approximately 3 days, the last day being occupied in the extraction of the charcoals. We have been able upon occasion to work up twice the quantity in this time in the laboratory.

Yields. We have now worked up some nine 14 lb. preparations and eight 28 lb. preparations by variants of this technique, and have given above the one which we consider to be most satisfactory. The $N/10$ HCl extract for 14 lb. contains between 1200–2000 doses of activity 0·4–1·0 mg. The alcoholic extracts which are coloured give a yield of some 1500 more doses of activity 1·0–1·5 mg. The second charcoal $N/10$ HCl extract gives about 600 further doses of between 1·0 and 2·0 mg. activity. The 50 % extract of the second charcoal is relatively inactive.

The highest yield which we have obtained from 14 lb. yeast has been 4400 doses. This is only some 70 % of the total doses present in the gum-free aqueous extract, which has given us a figure corresponding to 6000 doses in the 14 lb. This latter figure corresponding to one dose in 1 g. of pressed yeast is much higher than we had originally supposed, and than the figure given by Cooper [1912] of 2·5 g.

Though the yields are doubled as compared with our previous methods, and are more active at the same stage, it appears that there is still some considerable loss in the processes connected with the charcoal stage. Tests upon the filtrate from the charcoal have not given sufficiently reliable results to enable us to draw conclusions as to the amount of torulin present after the charcoal adsorption.

The presence of traces of metals and of a relatively large amount of acid is an objectionable feature to those who wish to employ concentrates prepared in this way for prolonged feeding experiments. These can be eliminated by removal of alcohol *in vacuo*, bringing to p_H 4·5 and then treating with H_2S. After this, the fluid is warmed upon the water-bath until flocculation of any colloidal sulphide takes place, filtered, and then boiled over the naked flame until free from H_2S. It is then evaporated to a small volume after addition of a small amount of HCl to drive off any acetic acid, and then subjected to the alcohol fractionation as described in the introduction to this paper (Exp. 1), but leaving out the lead acetate stage. In cases in which this technique has been applied the material has concentrated to an activity of 0·15–0·3 mg. a day, giving material of N content varying from 15–25 %.

The following alternative technique is suggested for use in feeding experiments, charcoal being used instead of mercury.

Alternative technique avoiding the use of mercury.

After removal of the gum with baryta, the barium is removed with sulphuric acid so as to leave only a minimal excess of sulphuric acid in solution. HCl is then added to p_H about 2·5 and barium sulphate removed as usual. 60 g. of norite charcoal is then stirred into the solution. This removes the bulk of the substances precipitable with mercuric sulphate leaving the torulin in solution at this p_H. After filtration, the solution is brought to p_H 7·0, and the usual charcoal extractions made. Upon the four occasions when this technique has been followed there has been a good yield in the $N/10$ HCl extracts of a high activity 0·3–0·5 mg., but the extra amount obtained in the 50 % acid alcohol extracts is poor. The method has much to recommend it to those who do not wish to obtain the highest possible yield of active material, but are satisfied to get a reasonable yield with the least trouble.

DISCUSSION.

It is hoped that the methods of obtaining reasonably active preparations of the curative substance with comparative ease may be of use to workers in many fields of nutrition. Now that such preparations can be obtained, there would seem to be no longer excuse for working with such impure mixtures as yeast autolysates as a source of this factor. It is not known yet what amount of torulin is needed in the nutrition of the rat, a matter which it is hoped to investigate shortly. This opens the necessity of discussing the place of torulin among the water-soluble vitamins, as also the excuse for the retention of this word. When this work was commenced, some years ago now, it was decided to adhere to the older curative test in spite of its many deficiencies because it seemed to be more likely to be testing for one factor than any test which involved the maintenance of weight or growth. Recent work upon the durability of vitamin B has tended to strengthen the view that this is so [see Harege and Carrick, 1926]. More especially Goldberger, Wheeler, Lillie and Rogers [1926] have shown that two factors are needed for the growth of rats, one of which is thermostable and present in autoclaved yeast, believed to be the pellagra-preventing factor, and both of which are of course present in fresh yeast. By the use of charcoal concentrates prepared by the early methods described by one of us [Peters, 1924], Chick and Roscoe [1927; Roscoe, 1927] have shown that the thermolabile factor for rat growth is present in the concentrates of the curative substance for pigeons (torulin concentrates).

It is therefore tempting to identify torulin with the antiberiberi vitamin, but this can at present be done only with reserve. It does not seem quite clear that symptoms of head retraction in the pigeon are certainly identical with beriberi [Eijkman, 1913], and it must be further remembered that preparations of an activity of even 0·1 mg. per day for the pigeon are still sufficiently impure to admit of the presence of more than one factor. Some evidence that more than one factor may still be present has been obtained already in collaboration with Miss Reader, in work which is now proceeding. The various workers who have been recently directing their attention to the problem of isolating the antineuritic vitamin would appear to be investigating different problems. Levene and van de Hoeven [1926] using the growth of rats for their tests have obtained preparations of which 0·08 mg. daily are required for the rat. Seidell [1926] uses the maintenance of weight in pigeons as a standard test. This test has also been employed by Funk and Paton [1922]. Both of these tests would seem likely to test for one factor, only so long as all the others are supplied in the diet or from stores accumulated by the animal.

In view of the uncertainty and differences of opinion as to the tests to be employed for the so-called antineuritic factor, it is probably best for the present to use the term torulin, as defined in the introduction to this paper.

Quite recently Jansen and Donath [1926, 1927] have described the isolation of a crystalline substance from rice polishings, making use of a protective test in birds. The substance described is protective to *Munia maja* (a small tropical bird) in doses of 0·002 mg. a day, and to the pigeon in doses of 0·01 mg. per day. This would be eight times more active than the preparation previously described by the authors [Kinnersley and Peters, 1925]. No mention, however, is made as to whether this substance is also curative. In connection with preventive tests, it will be remembered that Funk [1913] showed that even synthetic allantoin and hydantoin would lead to diminished loss of weight by pigeons upon polished rice, and also tend to delay symptoms of polyneuritis.

The interesting results of the Dutch workers led us to apply their methods to some of the 0·4 mg. a day material obtained in the course of this work. We are obliged, however, to record our disappointment, having been unable to concentrate the torulin present by the use of their technique, starting in one case at the silver stage, and in another at the platinum stage. It would appear therefore that their methods are not applicable to the concentration of torulin in these fractions. Nor can we obtain evidence that the Pauly reaction fractionates with the activity, material of activity 0·1 mg. giving a much less intense Koessler and Hanke [1919] reaction than the less active material from which it had been derived, when equi-dose amounts were compared. An amount equivalent to two torulin doses of activity 0·1–0·2 mg. per day gave a yellow reaction of less colour intensity than the reddish reaction given by 0·01 mg. histidine hydrochloride.

We lay no stress upon our failure to concentrate torulin in our fractions by a method applicable to the protective (? antiberiberi factor) in rice polishings. Doubtless it lies in some peculiarity of the yeast extracts[1]. It is, however, possible that the antiberiberi factor is distinct from torulin. The question at issue seems to be whether torulin is distinct from the antiberiberi factor, and what relation these properties of extracts bear to the protective factor in the sense of Jansen and Donath. It is indeed possible that the train of symptoms cured by torulin can only arise when absence of some other factor has accentuated a special phase of metabolism. This would explain certain abnormal responses to test doses, and would also justify the feeling that curative tests upon pigeons are not a reliable guide where human beriberi is concerned [Eijkman, 1913; Jansen and Donath, 1927].

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[1] Assuming that the curative substances in yeast and rice polishings are identical.

SUMMARY.

Improvements in methods of concentrating torulin are described, torulin being defined as the factor in yeast curative of symptoms of head retraction in pigeons induced by a diet of polished rice. By a removal of gum with baryta, subsequent procedures are simplified. Adsorption upon norite charcoal is not a property of torulin of activity 1·0 mg. per day, and is therefore due to a co-adsorbent in the earlier stages. The optimum p_H for adsorption upon norite charcoal is 7·0, provided that the extract has undergone a treatment with mercuric sulphate, but this is not true if this step be omitted. An improved technique for obtaining torulin concentrates of an activity of 0·2–1·0 mg. per day is described, based upon the above observations.

We are indebted to W. Wakelin for assistance both with the pigeons and the preparations. We are also grateful to Mr Carr of the British Drug Houses for his kind co-operation. One of us (H. W. K.) is indebted to the Medical Research Council for a personal grant, and we have to thank the Medical Research Council and The Christopher Welch Trustees for a grant for materials.

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CV. DIGESTION IN THE COCKROACH.

I. THE HYDROGEN ION CONCENTRATION IN THE ALIMENTARY CANAL.

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Although there is a considerable literature on the chemistry of digestion in insects this contains little more than records of the presence or absence of particular enzymes and there have been few attempts to investigate the properties of such enzymes more closely. The present work has been undertaken as a preliminary to the study of digestion in blood-sucking and disease-carrying insects; for it seemed desirable before dealing with such specialised material to make a more or less detailed study of the digestive enzymes in some insect of generalised structure and omnivorous habits, to compare the properties of these enzymes with those from other sources which have been the subject of detailed examination in the past and to relate these properties with the conditions under which digestion is normally carried on. It was hoped that such an investigation would serve as a useful point of reference in subsequent work, while at the same time the experiments have afforded an opportunity for the development of a technique for obtaining quantitative results with very small amounts of material.

The insect selected for this purpose was the small German Cockroach (*Blatella germanica*), but in most cases the experiments have been repeated and confirmed on the large American Cockroach (*Periplaneta americana*).

The present paper deals with the reaction of the intestinal contents of this insect and the factors by which this reaction is influenced.

THE REACTION OF THE INTESTINAL CONTENTS OF THE COCKROACH.

From the small amount of work which has been done on the reaction of the intestinal contents of insects [Crozier, 1924; Bodine, 1925; Senior-White, 1926], it appears that they do not as a rule show the extremes of acidity and alkalinity which are found in higher animals, though Jameson and Atkins [1921] record a p_H of 9·2 in the mid-gut of the larva of the silkworm.

The reaction of the crop contents of the cockroach has been variously stated to be weakly acid or neutral, the acidity when noted being usually attributed to changes in the food during digestion [Plateau, 1873]. Bodine

[1925], using the method of Felton [1921], measured the p_H of the gut contents of various grasshoppers (which belong to the same natural Order as the cockroach) and gives the p_H of the crop as 5·0–5·9 and of the mid-gut as 5·75–6·9, becoming less acid towards the hinder end. The p_H of the hind-gut and rectum varies from 6·4–7·2.

Methods.

The fluid available for the estimation of p_H in the gut of *B. germanica* amounts only to a few cubic millimetres. Approximate results may be obtained on this material by the rough method of Jameson and Atkins [1921]. But by the use of waxed plates and pipettes and the consequent elimination of the capillarity of the fluids a more exact colorimetric method has been developed.

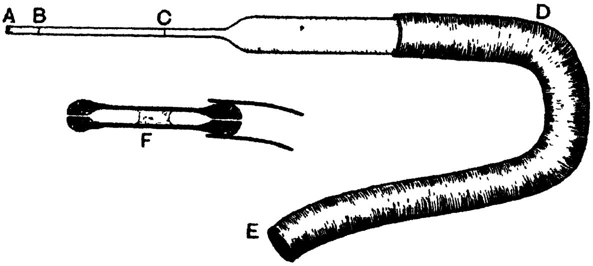

Fig. 1.

A capillary pipette (Fig. 1) is lined with a thin film of paraffin wax and two arbitrary graduations made at *B* and *C*, the volume *A–B* being about 2 mm.^{3}. The pipette is filled from *A–B* with the fluid to be tested, and then a solution of an appropriate indicator suitably diluted with boiled distilled water is run in until the fluid reaches *C*, the volume *B–C* being about 10 mm.^{3}. The accurate manipulation of these minute quantities of fluid requires a little practice, but is most readily effected as follows. A piece of rubber tubing is attached to the end of the pipette. The end *E* of this tubing is held in the teeth and the point *D* between the finger and thumb. Fluid is drawn in by compressing *D*, closing the teeth on *E*, relaxing *D* until the required volume has been sucked up and then releasing *E*. Fluid is expelled by the reverse process of closing *E*, compressing *D* and releasing *E* at the required moment. It is of course of the utmost importance *never to expel the fluid by blowing*. If the pipette has been well lined with wax and the fluid is expelled slowly and evenly the latter will be transferred quantitatively from the pipette. The fluid should be mixed by drawing it in and out several times.

The p_H of the fluid is determined by comparing it with mixtures made up with the same indicator solution in the same pipette, using standard buffer solutions in place of the test fluid in *A–B*. The colour comparison may be effected by expelling the drops of fluid on a white tile covered by a film of wax, where they form little spherical globules. But the evaporation of such drops is rapid and their exposure to the air is undesirable. A more satisfactory method of comparison is the following. A length of hard glass tubing of about 1·5 mm. bore is tested by means of a short column of mercury and those pieces of uniform bore are selected. It is then well washed with distilled water and tested for alkali by standing in a dilute solution of phenol red for 12 hours [Martin and Lepper, 1926]. Finally, the selected portions are dried, cut into lengths of about 1 inch and their ends sealed by dipping them in melted paraffin wax (*F*, Fig. 1). When required for use the ends are perforated with a fine needle. They are filled with the series of standard buffer mixtures or with the test mixtures by means of a rubber tube attached to one end, and the ends are then sealed by touching them with a heated glass rod. The closure of the ends of the tubes with paraffin caps has the twofold advantage of allowing them to be readily sealed and of enabling one to take up the fluid quantitatively from the waxed tile. Using the volumes indicated in the above description the column of fluid to be observed is about 5 mm. long.

With practice the whole procedure from the dissection of the insect to the colour comparison can be effected in half a minute. The standard buffers employed were of the Clark and Lubs series; colour differences of 0·1 p_H can be detected readily. The indicators used were the phenol red, bromothymol blue, bromocresol purple, methyl red and bromophenol blue of Clark and Lubs. The standards were made up fresh each day. The waxed tiles and other apparatus require very careful washing with distilled water between each experiment.

The method is a dilution method and as such is applicable only when the solution to be tested is adequately buffered. That this is the case with the saliva and gut contents of the cockroach was demonstrated as follows. A series of cockroaches fed upon different diets was dissected and the crop contents made up with suitable indicators as above described; but from each specimen several samples were taken. In one, the volume *A*–*B* would be filled with the test fluid; in another, it would be partly filled and made up to *B* with distilled water. In this way dilutions varying from 1:6 to 1:12 were compared. In the great majority of cases the results obtained were identical and in few cases did they differ by more than about 0·1 p_H unit.

A rough check on the results has been obtained by the method of Jameson and Atkins [1921] and, where the amount of fluid available has permitted, the results have been further controlled by using more than one indicator.

The dissection of the cockroach is very easily accomplished. The insect is anaesthetised with chloroform, laid on its back, and, while the body is steadied between the finger and thumb, the head is seized with forceps and gently drawn forwards. Almost invariably the salivary glands with the salivary receptacles distended with fluid and the entire gut with its contents as far back as the malpighian vessels may be drawn through the neck of the insect and placed upon the waxed plate. The various regions of the gut are then punctured and the escaping fluid rapidly taken up into the pipette.

Results.

Table I shows the results obtained from a series of freshly caught cockroaches which had been feeding on a varied mixed diet. The "saliva" in each case was the clear fluid distending the salivary receptacles.

Table I.

Region of gut	No. of estimations	Range of p_H	Average p_H
Saliva	22	6·7–7·4	6·9
Crop	12	4·6–6·0	5·2
Mid-gut	9	6·0–6·4	6·2

These results are in fair agreement with those obtained by Bodine on grasshoppers. There is a definite production of acid in the crop.

It was of interest to determine the source and nature of this acid. The digestion of the cockroach takes place mainly in the crop, the digestive juices coming from the salivary glands and the mid-gut. The reaction in both these regions is less acid than in the crop and this suggests that the acid is produced in the crop itself. The degree of acidity is too slight to be due to a strong acid such as hydrochloric and Gunzberg's test proved negative. Biedermann [1898], who noted a similar mild acidity in the mid-gut of mealworms, considered that this was due to an acid salt of phosphoric acid.

A series of *B. germanica* was fed on a mixture of 5 % gelatin and sucrose and dissected 5 hours later. The fluid from the crops of these insects was collected and showed a p_H of about 5·0. A portion of this fluid was mixed with appropriate quantities of Briggs' [1922] phosphate reagents in a capillary tube. but no trace of phosphorus could be demonstrated, although very small quantities can be detected by this method. On the other hand, the fluid gave a bright lemon yellow colour with dilute ferric chloride and there is little doubt that the acidity is due to lactic or other organic acids. It seemed probable that these acids are produced during digestion, and if such be the case the reaction should vary with the food taken. This point is the subject of the experiment given in Table II.

Table II.

Series	Food (mixed with water)	No.	p_H of crop		No.	p_H of mid-gut	
			Range	Average		Range	Average
A	Caseinogen	13	5·6–6·9	6·3	6	6·2–7·0	6·4
,,	Sucrose	10	4·4–5·6	4·8	7	6·2–6·4	6·3
B	Caseinogen	5	6·2–6·6	6·4	2	6·0–6·6	—
,,	Fibrin	2	5·8–6·4	—	1	6·2	—
,,	Gelatin	3	6·2–6·6	6·4	1	6·2	—
,,	Starch	3	5·2–5·8	5·5	—	—	—
,,	Maltose	2	5·6–5·8	—	1	6·4	—
,,	Lactose	2	5·6–6·0	—	—	—	—
,,	Sucrose	5	4·4–5·6	4·7	3	6·2–6·4	6·3
,,	Glucose	3	4·4–4·6	4·5	2	6·2	—

Series *A* starved for 2 days and dissected 3 to 18 hours after feeding. Series *B* given nothing but water for 20 days and dissected 4 to 6 hours after feeding.

It is clear from these figures that acid is being produced from carbohydrate and that this acid production is much more intense with sucrose and glucose than with the other carbohydrates. It will be shown in a subsequent communication that sterile extracts from the gut of the cockroach have no glycolytic action; it is therefore highly probable that the acidity is due to the activity of micro-organisms.

Microscopic examination of smears prepared from the crop contents of wild cockroaches showed a great variety of bacilli, cocci and small yeasts, and it was noted that yeasts were far more abundant in crops with a high acidity (p_H 4·6) than in those with a more nearly neutral reaction.

Finally, an emulsion of the crops of wild cockroaches was incubated with various sugars. The details of this experiment are given in Table III, and it will be seen that acid is produced most readily from glucose, less readily from maltose and least so from lactose. No gas was produced in any of the cultures. Stained films from the cultures showed the presence of yeasts, bacilli and streptococci.

Table III.

A. Equal quantities of an emulsion in distilled water of the crops of *B. germanica* incubated with 10 cc. of 2 % solutions of sugars and 10 drops of methyl red, under sterile conditions.
Controls (*B*) are the same using boiled emulsion.
Y = yellow to methyl red. The figures give the approximate p_H.

Time	Glucose *A*	Glucose *B*	Maltose *A*	Maltose *B*	Lactose *A*	Lactose *B*
11.0 a.m.	Y	Y	Y	Y	Y	Y
3.0 p.m.	5·2	Y	Y	Y	Y	Y
4·0 p.m.	4·8	Y	5·8	Y	Y	Y
9 30 a.m.	4·4	Y	4·6	Y	4·8	Y

DISCUSSION.

It is evident from the foregoing experiments that the acidity commonly observable in the crop of the cockroach is not a physiological constant for the animal but is dependent on bacterial action. None the less, since the cockroach feeds mainly on carbohydrates, a certain degree of acidity represents the normal reaction of that part of the gut where digestion mainly occurs; and no matter what the origin of this acid the digestive enzymes must be capable of acting over that range of p_H observed in the living animal. This subject will be considered in subsequent communications.

The variability of the reaction in the crop is in striking contrast with the relative constancy in the mid-gut. This is doubtless due to the neutralisation of the acid by the secretion of the mid-gut and perhaps also of the malpighian tubes. The reason for the failure of acid fermentation to continue in the mid-gut is not clear. Most probably it is due to the rapid absorption of sugar. For example, it was found that after a meal of sucrose the crop contents always reduced Fehling's solution strongly, whereas a much less intense reduction was obtained with the contents of the mid-gut. Nothing is known of the regulating mechanism of the sphincter of the proventriculus (gizzard) by which the passage of food from the crop to the stomach is controlled.

SUMMARY.

1. A colorimetric method for the determination of p_H in about 0·002 cc. of fluid is described. The essential feature of the method is the use of waxed plates and pipettes for the manipulation of the fluid.

2. In wild cockroaches (*Blatella germanica*) the average p_H of the saliva is 6·9, of the crop contents 5·2, and of the stomach 6·2.

3. The acidity of the crop is shown to be due to the action of micro-organisms, particularly yeasts, upon sugar. After protein food the average p_H is 6·3 in the crop and 6·4 in the stomach. After carbohydrate food these figures are 4·8 and 6·3 respectively.

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CVI. DIGESTION IN THE COCKROACH.

II. THE DIGESTION OF CARBOHYDRATES.

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THE most recent account of the digestive processes of the cockroach is that given by Swingle [1925], who employed the Common Cockroach (*Blatta orientalis*) in his experiments and whose conclusions are briefly as follows. The salivary glands produce only an amylase. The hepatic caeca of the mid-gut produce an invertase, maltase, lipase and protease. The remainder of the mid-gut (stomach) secretes the same enzymes though less abundantly. The protease he divides into a "trypsin" and an "erepsin"; but the tests employed for erepsin were (1) the formaldehyde titration of a peptone digest, and (2) the production in this digest of nitrogenous compounds not precipitated by phosphotungstic acid; both of which tests would be given equally well by trypsin alone. The hind-gut does not secrete digestive enzymes. The greater part, if not the whole, of digestion takes place in the crop, the enzymes passing backwards from the salivary glands and forwards from the mid-gut. The crop is lined by a membrane of chitin and is generally regarded as playing no part in the secretion of digestive ferments; but Swingle claims that extracts of washed crops contain traces of invertase, maltase and lipase, and the same claim is made by Sandford [1918] for lipase. Swingle does not decide from his evidence whether these enzymes are formed in the cells of the crop or absorbed by them from the digestive cavity.

It may be mentioned here that the results of Swingle have been confirmed, but that, in addition, an invertase has been found in the salivary glands of the German Cockroach (*Blatella germanica*), though not in the American Cockroach (*Periplaneta americana*) and that small quantities of lactase have been demonstrated in extracts of the mid-gut. With regard to the enzymes of the crop, the results of Swingle have been repeated by his method. The reliability of these results depends, however, upon the successful washing of the crops, a procedure which is not easily carried out, since the crops contain earthy material which is often very difficult to remove; and it has been shown that if the gut is washed out thoroughly in the living animal by giving it water but no food for a fortnight or three weeks, extracts of the crop are free from maltase and invertase, while the other parts of the digestive system retain their normal properties. These results conform to the more orthodox view that the crop plays only a passive rôle in digestion. The methods employed in these experiments will be described later when dealing with the enzymes in detail.

This paper will deal with those enzymes of the cockroach which are concerned in carbohydrate digestion, with a view to comparing them with the corresponding enzymes from other sources. It has been found [Wigglesworth, 1927] that these enzymes normally function in a medium rendered acid (p_H 5·0) by the action of micro-organisms. In the present communication it will be shown that they are adapted to work at this reaction; but that with this exception their properties are very similar to those of the enzymes with which they have been compared.

The most clear-cut distinctions between enzymes of a given kind are to be found in the effects of various ions upon their activity, and this part of the subject has received most attention here. The effects of substrate or enzyme concentration upon the kinetics of their action appear to be less important. Temperature effects are so dependent upon other conditions of the experiment that few clear comparative data are available, and this aspect of the matter has not been touched.

The comparisons which have been made are far from complete, but they are sufficient to indicate that the enzymes concerned in carbohydrate digestion in the cockroach are very similar to those of yeast and of higher organisms.

AMYLASE.

Amylases from various sources differ in their optimum p_H, in the effect of inorganic ions upon their amyloclastic and saccharogenic activity [Sherman and Tanberg, 1916] and in the ratio between these two measurements of activity under a given set of conditions [Sherman and Schlesinger, 1913]. The salivary amylase of man is characterised by being inactivated by dialysis, and by having its activity (both amyloclastic and saccharogenic) restored by the anions of salts in the following descending order: chlorides, bromides, nitrates, iodides, phosphates and sulphates. The optimum concentration of chloride is from 0·003 to 0·03 N [Willstätter, Waldschmidt-Leitz and Hesse, 1923]. The optimum p_H varies from 6·1 to 6·9, depending upon the salts present [Michaelis and Pechstein, 1914].

In the alimentary canal of the cockroach, amylase is confined to the salivary glands.

Methods.

Preparations of active enzymes. Stock preparations of enzymes from the cockroach have been obtained in two ways.

(i) Before dissection the insects are given nothing but distilled water for a week or a fortnight in order to clean out the alimentary canal. The gut is removed from the insect as previously described [Wigglesworth, 1927] and divided up into salivary glands, crop and mid-gut. These parts are collected from about twenty *B. germanica* and ground up in a small glass mortar with a little washed sand and a drop of glycerol until a fine uniform emulsion is obtained. This is then made up to about 10 cc. with 50 % glycerol to which are added a few drops of 5 % thymol in alcohol. This will be referred to as a "glycerol extract."

(ii) Dissection is carried out as before but the tissues are ground up with sand and a drop of water and of 5 % thymol in alcohol. The resulting emulsion is dried at room temperature and powdered. This will be referred to as a "dry preparation."

Human ptyalin was obtained by holding in the mouth a mixture of equal parts of glycerol and water and filtering the mixture.

General method of experiment.

(i) *Amyloclastic experiments.* The reactions are carried out in small test-tubes (100 mm. by 6 mm.), the total volume of fluid present being 1 cc., of which 0·5 cc. consists of a 2 % solution of soluble starch. A small pipette graduated in divisions of 0·025 cc. is placed in each tube at the outset of the experiment. A water-bath at 37° has been used throughout. At suitable intervals 0·025 cc. is withdrawn and run into 0·5 cc. quantities of very dilute (almost colourless) acidified iodine solution measured out into a series of uniform test-tubes. When the optimum of a series of conditions is sought, the method of Michaelis [1922] is adopted, *i.e.* the series of reacting mixtures is tested at an equal interval from the outset of the experiment and the one showing the greatest degree of change taken to represent the optimum. When the relative activity over a range of conditions is sought, the time taken to reach a suitable mauve tint in the fastest tube is noted. This tint is then used as an arbitrary end-point and the time taken by each mixture to reach this colour is observed. The reciprocals of these respective times are taken to represent velocity of action and, expressed as percentages of the fastest rate, are reported in the various experiments as "relative velocity." An additional check on the results has been obtained by noting the time taken to reach the achromic point. But for general use the former method has proved more satisfactory because in the slowly reacting mixtures the achromic point may never be attained, while in the rapidly reacting mixtures there is nothing to indicate whether it has been reached some time past.

Where two enzyme preparations are compared under the same series of conditions, the concentration of each is so adjusted before the experiment that it shows approximately the same activity as the other when both are at their respective optima. It is most convenient if the arbitrary end-point is reached in the fastest tube in about 15 minutes.

(ii) *Saccharogenic experiments.* The experiments are carried out precisely as above except that pipettes graduated in 0·1 cc. divisions are employed and the sugar produced in a given time is estimated by the method of Hagedorn and Jensen [1922][1]. The concentration of enzyme is adjusted to give about 0·25 % of "glucose" in 15 minutes in the fastest mixture. The blank on such mixtures is so small that no preliminary precipitation is necessary. The amount of phosphate present in the phosphate buffer mixtures has been shown to have no effect on the reducing power of sugars as estimated by this method [cf. Visscher, 1926]. In these experiments it is assumed that the velocity is directly proportional to the amount of reducing sugar (expressed as "glucose") which is produced in a given time. This expression assumes that the hydrolysis of the starch is a linear function of time, an assumption which is probably only approximately true and which is only justified because no far-reaching conclusions are based upon it.

Results.

(i) *The effect of salts.* An experiment showing the effect of salts on cockroach amylase is given in Table I.

Table I.

Mixtures in reaction-tubes made up as follows: 0·1 cc. glycerol extract of salivary gland of *B. germanica* + 0·1 cc. 0·5 *M* solution of salt + 0·3 cc. distilled water + 0·5 cc. 2 % soluble starch.

Salt	Relative activity (amyloclastic)
None added	30
Sodium sulphate	47
Sodium phosphate (p_H 6·8)	50
Sodium nitrate	74
Potassium iodide	80
Sodium bromide	100
Sodium chloride	100

This experiment shows that the amyloclastic power of cockroach amylase is influenced by salts in the same way as ptyalin. In an experiment where the saccharogenic activity was measured, this was found to be influenced in the same way by the addition of salts.

A dry preparation of cockroach salivary gland was extracted with distilled water for 24 hours and the extract dialysed in a small collodion sac, prepared by the method of Gates [1922], against several changes of distilled water for 24 hours. The 2 % starch solution was similarly dialysed. In the absence of added salt the preparation so treated was almost inactive. A comparison of amyloclastic activity at various concentrations of sodium chloride was then made. These results are given in Fig. 1 and show an effect identical with that described in the case of human ptyalin. It is of interest to note that the amount of enzyme present in each of these mixtures was equivalent to one-fiftieth of the salivary gland of the cockroach.

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[1] Since Hagedorn and Jensen make no reference to the fact and as I have found so many workers unacquainted with it, I may mention here that the *N*/200 solution of sodium thiosulphate will remain perfectly stable in ordinary distilled water for many weeks if this be made just alkaline with sodium hydroxide. Thus the titre of a solution of thiosulphate in distilled water against standard potassium iodate changed from 18·0 to 22·4 in 17 days, whereas a similar solution made just alkaline with sodium hydroxide changed from 18·1 to 18·2 in 2 months.

(ii) *The effect of hydrogen ion concentration.* In considering the "adaptation" of an enzyme to its environment, the precise concentration of hydrogen ions necessary for its optimal activity is of less importance than its ability to function over that range of p_H which it is liable to encounter in its sphere of action. It is on this account that in the experiments here reported more attention has been paid to the relative activity of the enzymes over a wide range of p_H than to the determination of the absolute optimum. Moreover, differences in activity around the optimum are so slight that a more reliable basis of comparison is afforded by considering the p_H at which activity is reduced by 50 %, irrespective of any theoretical interpretations [Michaelis and Davidsohn, 1911] which may be put upon this figure.

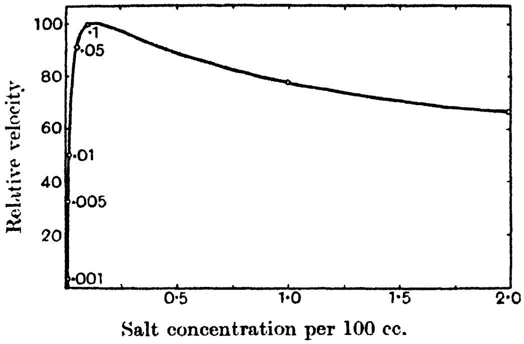

Fig. 1. Effect of sodium chloride on dialysed amylase from the salivary gland of the cockroach.

The glycerol extracts prepared as above described are practically unbuffered and the required hydrogen ion concentration has been obtained by taking in each tube 0·1 cc. of glycerol extract (after dilution if necessary), 0·1 cc. 1 % sodium chloride and 0·3 cc. of buffer. 0·5 cc. of 2 % soluble starch is added at the last moment. The phthalate and phosphate buffer mixtures of Clark and Lubs have been used. (Borate buffers cannot be employed in the presence of glycerol because of the production of the highly dissociated glyceroboric acid.) The p_H has been re-determined colorimetrically at the end of the experiment and shown to be unchanged.

Under these conditions the optimum p_H for cockroach amylase was found to be about 5·9 and in the case of human ptyalin about 6·5.

The relative activity over a wide range of p_H is shown in Fig. 2, which was obtained from a preparation from *Blatella germanica.* From this it will be seen that whereas the cockroach amylase still shows 50 % activity at p_H 4·8, human ptyalin is almost inactive at this reaction. The cockroach enzyme is much more tolerant of acid, a finding which was to be expected from the conditions obtaining in the gut of this insect [Wigglesworth, 1927]. The relative difference in activity in the alkaline direction is less striking. Twelve hours after the conclusion of this experiment digestion was still incomplete in the more acid mixtures. The p_H in the various mixtures was brought back to about 6·4 with potassium hydroxide and acetic acid, and 0·5 cc. of 2 % starch added. In the tubes at p_H 4·0 both amylases had been destroyed. At less acid reactions the enzymes had been partially destroyed, that of the cockroach being the more resistant. Clearly the diminished activity in the acid region is due partly to enzyme destruction and partly to a reversible inactivation.

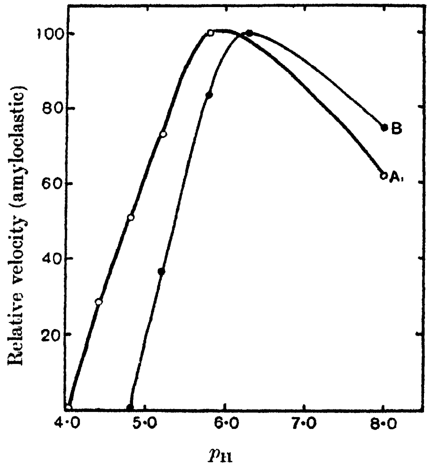

Fig. 2.

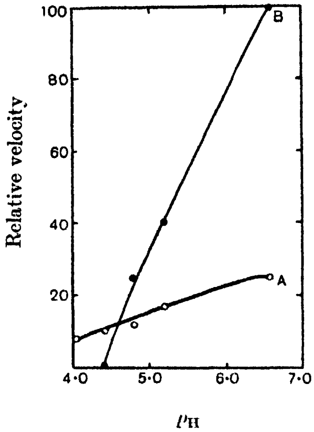

Fig. 3.

Fig. 2. p_H-activity curves (amyloclastic) for amylase. *A*, Salivary amylase of *Blatella germanica*. *B*, Human ptyalin.

Fig. 3. p_H-activity curves for amylase. *A*, Salivary amylase of *Periplaneta americana*. *B*, Human ptyalin.

Fig. 3 shows a similar experiment with an amylase preparation from the salivary gland of *Periplaneta americana*. Owing to an error in calculation the human preparation was nearly four times as active as the cockroach preparation at about the optimum; yet the activity of the cockroach amylase falls off far less rapidly in the acid direction.

Fig. 4 is a comparison of the two p_H-activity curves in an experiment where sugar production was taken as a measure of activity. This gives exactly the same results as the amyloclastic experiments.

It was of interest to determine whether the greater resistance of the cockroach amylase to acid was due to differences inherent in the enzyme or to some associated factor; for it is well known that the optimum p_H of amylases is markedly affected by the presence of salts [Michaelis and Pechstein, 1914]. This point was tested in the experiment given in Table II. Preparations of ptyalin (*A*) and of amylase from the German Cockroach (*B*) were adjusted to have the same activity at their p_H optima. Mixtures were made up as shown in the table and their activity compared at p_H 4·4. The "inactivated" enzymes were left for 5 minutes in a boiling water-bath and were shown to be completely inactive.

Table II.

Mixture	Relative activity
0·1 cc. *B* + 0·1 cc. "inactivated" *A* + 0·1 cc. 1 % NaCl + 0·2 cc. phthalate buffer p_H 4·4 + 0·5 cc. 2 % starch	100
0·1 cc. *B* + 0·1 cc. 50 % glycerol: the rest as before	100
0·1 cc. *A* + 0·1 cc. "inactivated" *B*: the rest as before	12·5
0·1 cc. *A* + 0·1 cc 50 % glycerol: the rest as before	Very slight

A similar result has been obtained in the case of the amylase from the American Cockroach, and would seem to indicate that the ability of the cockroach enzyme to function in a more acid medium is due in part at least to some associated substance which is not destroyed by heating at 100° for 5 minutes.

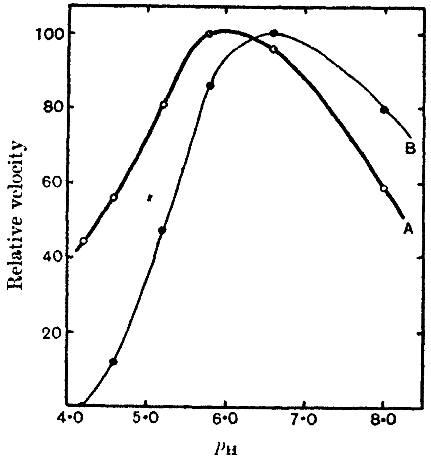

Fig. 4.

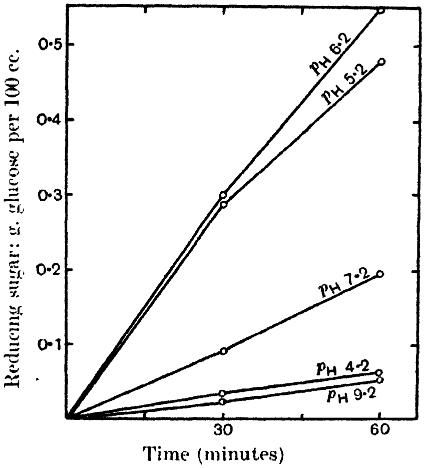

Fig. 5.

Fig. 4. p_H-activity curves (saccharogenic) for amylase. *A*, Salivary amylase of *Blatella germanica*. *B*, Human ptyalin.

Fig. 5. Hydrolysis of sucrose by cockroach invertase.

(iii) *Ratio of amyloclastic to saccharogenic activity.* Preparations of cockroach amylase and human ptyalin were adjusted to have about the same activity at p_H 6·6; and, preparing the mixtures in the usual manner, their amyloclastic and saccharogenic activities were compared at p_H 6·6 and 8·0. The ratio of amyloclastic to saccharogenic activity was arbitrarily expressed as $\frac{1}{t} : x$; where t is the time in minutes required to reach the arbitrary end-point of starch hydrolysis and x is the reducing sugar (expressed as g. "glucose" %) produced in 15 minutes. The results are shown in Table III.

Table III.

Enzyme	p_H	Ratio of amyloclastic to saccharogenic activity
Cockroach	6·6	1 : 2·5
,,	8·0	1 : 2·3
Human ptyalin	6·6	1 : 3·2
,,	8·0	1 : 3·3

Sherman and Schlesinger [1913] found that this ratio (expressed in other units) differed widely in various enzymes, ranging from 9 : 1 to 2 : 7. They found that whereas preparations of pancreatic amylase showed a more or less constant ratio, the ratio in the case of plant amylase preparations was extremely variable. Although a long series of experiments would be necessary to establish the point completely, the above results suggest that by this criterion human and cockroach amylases are similar in nature.

INVERTASE.

Of the enzymes which hydrolyse sucrose, that obtainable from yeast has been the most closely studied. This enzyme is reversibly inactivated by alkali, and the alkaline branch of the p_H-activity curve is regarded by Michaelis and Davidsohn [1911] as the dissociation-residue curve of a weak-acid enzyme which is active only in the form of undissociated molecules. The acid branch of the curve is similarly interpreted by Myrbäck [1926]; but in this region there is in addition the factor of irreversible destruction of the enzyme. The activity of yeast invertase is inhibited by heavy metals, by high concentrations of sodium chloride and by glycerol among other substances.

Euler and Svanberg [1921] studied the invertase of the human jejunum and found that it was active in a more alkaline medium than yeast invertase; and Euler and Myrbäck [1921] showed that this human invertase was more sensitive to heat.

Sarin [1923] observed that the invertase from the gut of the honey-bee was inhibited by glycerol. Nelson and Cohn [1924] made a careful comparison between the invertase of honey and of yeast. They found certain differences in the kinetics of the reaction in relation to substrate concentration, but the optimum concentration of sucrose for both enzymes was about 4 %. The optimal range of p_H was 3·5–5·0 in the case of yeast and 5·5–6·3 in the case of honey invertase, the same relative change in p_H having a greater effect upon the latter enzyme.

The distribution of invertase in the alimentary tract of the cockroach has already been described.

Methods.

The activity of invertase has been measured by the same method as the saccharogenic activity of amylase, 3 % sucrose being used as substrate. The concentration of enzyme has usually been adjusted to give rise to about 0·25 % reducing sugar (measured as "glucose") in 1 hour.

Under these conditions the hydrolysis of sucrose has been shown to be practically a linear function of time (Fig. 5), and the activity of the enzyme may be regarded as directly proportional to the amount of reducing sugar produced at the end of half an hour. This relation obtains around the optimal p_H of the enzyme and on the alkaline side of this; but on the acid side (see

p_H 4·2 and 5·2 in Fig. 5) the activity soon begins to diminish as the result of irreversible destruction of the enzyme and the relative activity will apparently fall off as the experiment proceeds. In other words, in short experiments with an active preparation the optimal zone will extend further in the acid direction. Hence the importance of so adjusting the concentration of the enzymes to be compared that they act at approximately the same rate.

Yeast invertase was prepared by grinding up fresh yeast with 50 % glycerol and a little fine sand. Human invertase was prepared by extracting for a week, with 50 % glycerol, the mucous membrane removed from the jejunum and then filtering.

The various preparations were incubated for 15 hours with a 0·5 % solution of glucose. There was no change in reducing power. None of the preparations contained glycolytic enzymes.

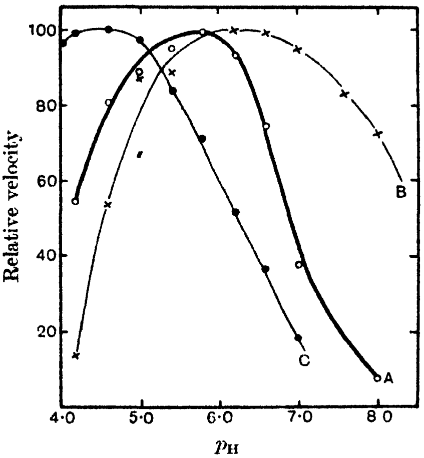

Fig. 6.

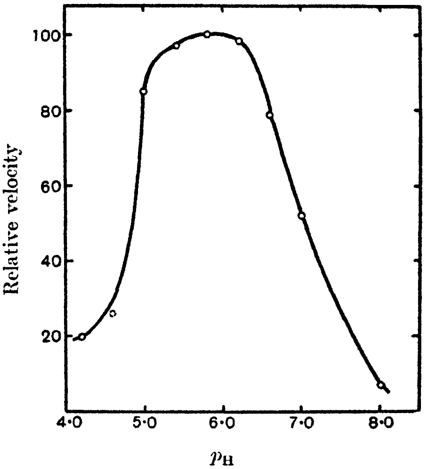

Fig. 7.

Fig. 6. p_H-activity curves for invertase. *A*, Mid-gut invertase from *Blatella germanica*. *B*, Invertase from human jejunum. *C*, Invertase from yeast.

Fig. 7. p_H-activity curve for invertase from the salivary gland of *Blatella germanica*.

(i) *The effect of salts, heavy metals and glycerol.* Low concentrations of sodium chloride have no effect upon the activity of cockroach invertase. A concentration of 1 % has an inhibitory action (Table IV).

Table IV.

Invertase from mid-gut of *B. germanica*.

NaCl concentration (g. %)	Relative activity
Nil	100
0·1	100
1·0	61

That glycerol when present in a concentration above 10 % has a strong inhibitory effect in spite of the increased quantity of enzyme present is shown in Table V.

Table V.

Glycerol extract of mid-gut of *P. americana*.

cc. of enzyme preparation	% glycerol	Relative activity
0·2	10	100
0·4	20	88

Cockroach invertase is extremely sensitive to silver nitrate. This is shown in Table VI.

Table VI.

Equal quantities of invertase (glycerol extract) from mid-gut of *B. germanica*.

Concentration of silver nitrate	Relative activity
Nil	100
0·0001 *N*	46·5
0·001 *N*	0

In all the above respects cockroach invertase behaves like yeast invertase.

(ii) *The effect of hydrogen ion concentration.* The methods employed were the same as those already described in connection with amylase. It was shown that at p_H 6·0 the activity was the same whether phosphate or phthalate buffer was used.

Fig. 6 shows p_H-activity curves for the invertase from yeast, from the cockroach and from the human jejunum. The curve for the cockroach enzyme is very similar to that obtained by Nelson and Cohn [1924] for honey invertase. The curves for yeast invertase and human invertase agree with those of Michaelis and Davidsohn [1911] and Euler and Svanberg [1921] respectively. It is clear that cockroach invertase is well adapted to work at the range of reaction (p_H 4·4–6·2) commonly found in the crop. As was noted by Nelson and Cohn in the case of honey invertase the zone of p_H over which it is active is more restricted than that of the other two enzymes.

At the end of this experiment the tubes were left in the water-bath for about an hour and then the p_H of the mixtures at 4·2 and 8·0 was adjusted to 5·8 by the addition of traces of 5 % caustic potash and of glacial acetic acid respectively (*vide infra* in connection with maltase). These mixtures were incubated for a further period of 30 minutes; and it was found that whereas the mixture previously at p_H 8·0 showed 100 % activity, that which had stood at p_H 4·2 was almost inactive, *i.e.* the enzyme is stable but inactive on the alkaline side of its optimal zone, while it is highly labile on the acid side—a behaviour identical with that of yeast invertase.

Fig. 7 shows the p_H-activity curve of invertase from the salivary gland of the German Cockroach. This curve falls off a little more rapidly in the acid direction but in other respects is the same as that described above.

MALTASE.

Maltase has not been so extensively studied in the past as invertase. The maltase of yeast was shown by Michaelis and Rona [1913] to have a narrow p_H range of 6·1–6·8 and to be very sensitive to changes in reaction outside this zone, whether in the acid or the alkaline direction, and to be in general a far more labile enzyme than invertase. It is readily adsorbed by kaolin, while invertase is not, and is readily extracted from this adsorbate with secondary alkali phosphate [Willstätter and Kuhn, 1921]. Its action is inhibited by glycerol and by sodium chloride above 0·2 N concentration. The properties of the maltase of the succus entericus do not seem to have been closely studied.

In the cockroach, maltase occurs only in the secretion of the mid-gut.

Methods.

Quantitative measurements of maltase activity have been made by the method of Hagedorn and Jensen (as described above), using 0·25 % maltose as substrate. By adding measured quantities of a standard solution of glucose to measured quantities of this substrate, a graph was prepared from which the reducing power of the mixtures in g. glucose %, as calculated from the table of Hagedorn and Jensen, could be converted into "percentage hydrolysis" of the maltose substrate. Serial experiments were usually continued until about 60 % hydrolysis had occurred in the fastest tube. In experiments on the p_H range of the enzymes, the p_H has been plotted against this "percentage hydrolysis." When such a large fraction of the substrate is hydrolysed, this percentage can give only an approximate indication of the "relative velocity" of action.

The first attempts to demonstrate maltase in the glycerol extracts of cockroach organs were unsuccessful. It seemed probable that the enzyme had been adsorbed by the ground sand used in the preparation. The extract was therefore poured off and the sediment shaken for about 2 hours at 30° with a few cc. of phosphate buffer p_H 6·8. This extract was very rich in maltase. In the experiment given in Fig. 8, for example, the enzyme equivalent to one-hundredth of a cockroach was present in each mixture and this effected 60 % hydrolysis of 1 cc. of 0·25 % maltose in an hour. Much of this activity was lost in the course of about a week at room temperature. The experiments recorded below were made upon such an extract or upon a similar extract made from the "dry preparations" of tissues.

For experiments on yeast maltase, an aqueous extract of dried yeast was employed. In the case of human maltase, the same preparation as for invertase was used, but this was suitably diluted to obviate the inhibitory action of the glycerol.

Table VII.

Test mixture	Percentage hydrolysis after 1 hour
No added salt or glycerol	37·0
0·1 % sodium chloride	37·5
1·0 % sodium chloride	8·0
10 % glycerol	0·0

(i) *The effect of sodium chloride and glycerol on cockroach maltase.* This effect is shown in Table VII. The action of maltase is partially inhibited by

1 % sodium chloride and completely inhibited by 10 % glycerol. It is more susceptible to glycerol than is invertase. In this it resembles yeast maltase; and this fact affords a further reason for the failure to demonstrate maltase in the glycerol extracts of cockroach tissues.

(ii) *Effect of hydrogen ion concentration on cockroach maltase.* Since the extracts containing maltase were made with phosphate buffer, special methods had to be adopted to obtain the required p_H. The experimental mixtures were made up as follows: 0·1 cc. of enzyme preparation, 0·2 cc. of buffer at the required p_H and 0·2 cc. of distilled water. A duplicate series of mixtures was made up in uniform tubes as follows: 0·2 cc. of the same series of buffers as before, and 0·3 cc. of distilled water. Suitable and identical amounts of appropriate indicators were added to the two series of mixtures; and the mixtures containing enzyme were adjusted to the same p_H as the standards without altering their volume appreciably in the following manner. The ends of two capillary tubes were drawn out into hair-like capillaries and filled with 0·5 *N* HCl and 0·5 *N* NaOH respectively, to such a level that when dipped in liquid the reagent would flow gently out. The test mixtures were stirred cautiously with one or other of these capillaries until the colour matched that of the standard. 0·5 cc. of 0·5 % maltose was then added and the experiment continued as previously described.

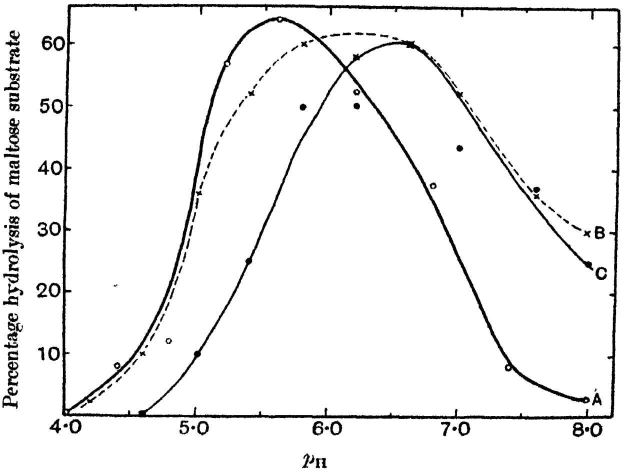

Fig. 8. p_H-activity curves for maltase. *A*, From mid-gut of *Periplaneta americana*. *B*, From human jejunum. *C*, From yeast.

Fig. 8 shows the "percentage hydrolysis" of maltose by the three enzymes plotted against p_H. These curves give only an approximate indication of the relative activity at the different reactions. The curve for cockroach maltase (with an optimal zone from 5·0 to 6·2) falls off more rapidly than that for invertase in the acid direction, but it is active over the greater part of the p_H range found normally in the crop of the insect. The curve for yeast agrees well with that obtained by Michaelis and Rona [1913]. Human maltase is active in the acid direction as far as is the cockroach enzyme, but it is much more resistant than the latter on the alkaline side.

Lactase.

Relatively few comparative studies have been made upon lactase. The lactase of yeast is said to have its optimum p_H at 7·0.

The presence of lactase was demonstrated in extracts from the mid-gut of the cockroach, but not in those from salivary glands or crop. The formation of glucosazone and the increase in reducing power of a lactose solution, as measured by the method of Hagedorn and Jensen, were used as qualitative tests. The enzyme was obtained by extracting "dry preparations" of enzymes from the mid-gut with phosphate buffer at p_H 6·8. For the p_H-activity curve given in Fig. 9, the methods described under maltose were employed, both for the calculation of the percentage hydrolysis and for the adjustment of the p_H. The enzyme was far less active than the maltase in the same preparations. For example, in this experiment the enzyme equivalent of one-tenth of a cockroach was employed in each mixture and these were incubated for 15 hours. The range of p_H over which it is active is similar to that of the enzymes described above, the optimal zone being from 5·0 to 6·4.

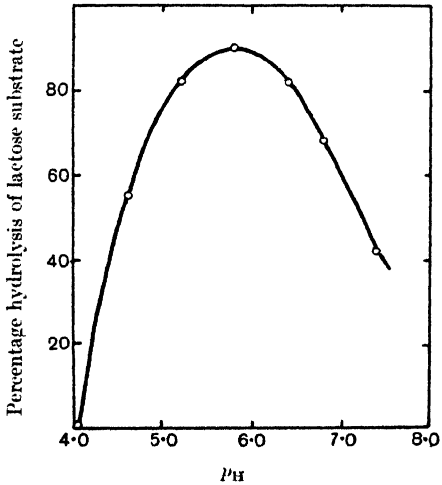

Fig. 9. p_H-activity curve for lactase from mid-gut of *Periplaneta americana*.

This lactase was shown to be completely inhibited by 10 % glycerol.

Neither the yeast preparations nor those from the human jejunum contained lactase.

Discussion.

Although, in the experiments here described, not all the known properties of the various enzymes have been compared, yet these experiments are sufficient to indicate the existence of a very close similarity between the enzymes of the cockroach and those from yeast and from man. As was to be expected from the reaction of the crop contents of the cockroach, the enzymes of this insect are most active over an acid range from about p_H 5·0 to 6·5; and this range is similar for all the enzymes except amylase, in which it extends in the alkaline direction to p_H 7·0. It should be noted that the conditions under which this enzyme works are peculiar in that besides acting with the other enzymes in the crop, the saliva is expelled from the mouth of the insect and mixed with the food before it is ingested.

The results have been got from very small quantities of material; but the good agreement with the results of others, obtained when dealing on the same scale with the other enzymes, is evidence that they are reliable.

SUMMARY.

1. The salivary glands of the cockroach secrete an amylase and in *Blatella germanica* (though not in *Periplaneta americana*) an invertase. Extracts of the crop, when thoroughly freed from food contents, do not contain enzymes acting on carbohydrates. The stomach and hepatic caeca secrete invertase, maltase and small quantities of lactase.

2. The amylase of the cockroach is inactivated by dialysis and is affected by salts in the same way as is human ptyalin. The ratio of amyloclastic to saccharogenic activity is similar to that of human ptyalin. The p_H-activity curves for these two enzymes have been compared. Under the same conditions cockroach amylase is more resistant to acid (optimum p_H 5·9). This is shown to be due in part at least to some associated factor which is not destroyed by heating at 100° for 5 minutes.

3. Cockroach invertase has been compared with yeast invertase and human jejunal invertase. It is partially inhibited by 1 % sodium chloride, 20 % glycerol and 0·0001 *N* silver nitrate; and completely inhibited by 0·001 *N* silver nitrate. The p_H-activity curve for cockroach invertase shows an optimal range from 5·0 to 6·2 which is intermediate between the ranges for yeast invertase and human jejunal invertase.

4. Cockroach maltase has been compared with the maltase of yeast and the human jejunum. It is partially inhibited by 1 % sodium chloride and completely inhibited by 10 % glycerol. It is adsorbed by powdered sand, but can be extracted from this with phosphate buffer p_H 6·8. The p_H-activity curve shows an optimal range from 5·0 to 6·2, which is more acid than the range in the maltase of either yeast or human jejunum.

5. Cockroach lactase is completely inhibited by 10 % glycerol and shows an optimal zone of p_H from 5·0 to 6·4.

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CVII. THE PRODUCTION OF LACTIC ACID IN FROG'S MUSCLE *IN VIVO.*

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(Received June 29th, 1927.)

ALMOST all the work which has been done upon the production of lactic acid in muscular activity (Fletcher and Hopkins, Meyerhof, etc.) has been carried out upon muscles removed from the animal body and consequently deprived of what must certainly be a potent factor in the recovery process *in vivo*, viz. the circulating blood. The experiments here described were carried out in order to see to what extent lactic acid produced by the activity of the living animal would accumulate in the muscles.

The work was begun in 1922 and as there is no prospect of our being able to complete the investigation we have considered it worth while to publish it in its present stage.

If a frog be placed in a large vessel of water at 35–40° it swims round with great violence and after a period of about 1 to 2 minutes it exhibits a jerky spasmodic movement of its legs as though the muscles were unable any longer to respond to the nervous stimuli being sent into them. Shortly after this stage all movement ceases and the frog becomes stiff and unconscious. But if, now, the animal be removed from the hot bath it will recover consciousness in a few minutes and gradually reacquire the power of movement in its limbs, though frequently the muscles do not appear to have recovered completely for at least half an hour. The various stages of this process were used for investigating the production and removal of lactic acid.

The following are typical observations made upon frogs under these conditions.

At 33°. Frog became weaker but could still swim about after being in the water up to 5 minutes.

At 35°. Frogs came to rest after 3 minutes and when taken out they seemed quite limp and toneless (cf. at 38°) until breathing and movements returned and then the tone seemed to return to the muscles all at once.

At 37°. Came to rest in 1 minute. Taken out at once. Eyes closed. Breathing started after 1 minute. Eyes slowly opened and breathing quickened. Legs not nearly so fixed as at 38° but still tend to return to a semi-extended position. Complete recovery after 2 minutes.

At 38°. (i) Frog swims about much more violently than at the lower temperatures tried. Stiff after 1 minute. Taken out at once. Legs stopped in a semi-extended position and returned to this point whether forcibly flexed or extended. Could first move legs very weakly after 2 minutes. Could first hop effectively after 4 minutes. Complete recovery in about 15 minutes.

(ii) Stiff after 1 minute. Left in bath 30 seconds longer. Breathing recommenced after about 1½ minutes. Reflexes in hind legs began to appear after 3½ minutes but could not be repeated at short intervals. Front legs remained stiff. Remained very sluggish for upward of 20 minutes. Breathing as in the normal frog during this time. Could not jump properly. Could not execute many movements properly. At end of 2 hours recovery in all limbs was complete and frog appeared normal in every way.

The observations on the lactic acid content of the muscles have been made only at 38°, the frog being left in the bath in each case until stiff, *i.e.* about 1 minute. The lactic acid was estimated by the micro-method of Meyerhof [1920], the muscle being removed as rapidly as possible from the hind legs of the frog used and one estimation made on each leg.

Procedure	g. lactic acid per 100 g. muscle	
"Resting minimum" of normal frog	0·031	0·031
Muscle removed from frog and kept at 40° for 1 hour. ("Lactic acid maximum")	0·406	—
Muscle removed from frog and kept at 40° for 1 hour. ("Lactic acid maximum")	0·443	—
Pithed frog in bath at 38° for 1 min. 30 secs.	0·042	0·044
Active frog left in bath at 38° until stiff	0·112	0·125
,, ,, ,,	0·125	—
,, ,, ,,	0·112	0·113
,, ,, ,,	—	0·176
,, ,, ,,	—	0·125
Frog in bath as above, after recovery at room temperature for 18 minutes	0·107	0·111
,, ,, ,, ,, 20 ,,	0·091	0·096
,, ,, ,, ,, 32 ,,	—	0·070
,, ,, ,, ,, 30 ,,	0·039	0·045
,, ,, ,, ,, 60 ,,	0·037	0·040

It will be seen that at the time when the animal is unable any longer to bring about the contraction of its muscles, the latter contain a high percentage (average 0·125 %) of lactic acid. Meyerhof [1920] showed that the gastrocnemius of the frog, removed from the body and stimulated indirectly by means of a tetanising current until it could no longer respond, attained a concentration of lactic acid of about 0·19 %. Hence it is not improbable that the movements of the frog are arrested by the lactic acid accumulating under the conditions of our experiments more rapidly than it can be removed.

If the frog is pithed and left in the hot bath for a corresponding period, there is very little more lactic acid present than the normal resting minimum (0·043 % as against 0·031 %). The lactic acid is, clearly, the result of muscular activity.

The figures also show that the removal of this accumulated lactic acid is very slow. It is often not completely removed in half an hour, though after the lapse of an hour the resting minimum value has been practically regained.

The large lactic acid production and the slowness of the recovery process under these conditions should make this material very suitable for studies on lactic acid in the living organism.

SUMMARY.

1. When placed in a bath at 38° the frog is at first violently active, but becomes stiff and motionless in about 1 minute.

2. In this state the muscles of the hind legs show an average content of lactic acid of 0·125 %.

3. The muscles of a pithed frog under similar conditions contain little more lactic acid (0·043 %) than the normal resting minimum (0·03 %).

4. On removal to room temperature the frog recovers in a few minutes, but the lactic acid content of the muscles does not regain the normal level until half to one hour later.

We wish to express our thanks to Sir F. G. Hopkins for suggesting this problem to us and for his helpful interest in the work. The research was carried out during the tenure by each of us of a Frank Smart Studentship of Gonville and Caius College and of a grant from the Board of Scientific and Industrial Research.

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CVIII. THE TITRATION OF PROTEIN HYDROLYSATES.

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Foreman [1920] and Harris [1924] have shown that the acid radicles of amino-acids can be titrated quantitatively if the K_a of such amino-acids in solution were increased sufficiently to afford a sharp reaction with the indicators used. This was effected by titrating in strong alcoholic solution (above 87 % alcohol) to phenolphthalein in Foreman's method and to thymolphthalein in Harris's method. Better results were obtained by the addition of formaldehyde to the alcoholic solution according to Sørensen's method [1907]. Also, by the inclusion of formaldehyde in the solutions, difficulties in obtaining accurate results with dicarboxylic acids and diamino-acids were overcome.

The procedure was to neutralise the *aqueous* solutions to the respective indicators and, after adding the requisite volume of neutralised solvent to the solution, again to neutralise to the indicator used. This increase in acidity was the basis for calculating the acid radicle present in the solutions. Work was done on most of the common amino-acids individually so that data of the behaviour of all the different types of amino-acids under such conditions have been collected.

Attention has been paid in the present work to the actual distribution of nitrogen in solutions containing mixtures of amino-acids and not to the total acid radicle present. The principle of weight of nitrogen equivalent to titratable acid radicle has, therefore, been considered. With a solution of glycine hydrochloride, the hydrochloride portion would be neutralised completely in aqueous solution with alkali to phenolphthalein, whereas in the subsequent alcoholic titration, the carboxyl group would be quantitatively neutralised. The titre of the carboxyl group would be exactly equivalent to the weight of nitrogen present in the glycine solution, *i.e.* every 1 cc. of normal alkali used in the alcoholic titration would be equivalent to 0·014 g. of nitrogen.

From Foreman's paper [1920] the following deductions have been drawn concerning the nitrogen of the various amino-acids equivalent to the titratable value of such acids in alcohol-formalin solution after previous neutralisation in aqueous solution:

Monoamino-acids.	All the nitrogen.
Dibasic acids.	All the nitrogen (one carboxyl group is neutralised in aqueous solution).
Cystine and lysine.	All the nitrogen.
Arginine.	One-quarter of the nitrogen.
Histidine.	One-third of the nitrogen.

Ammonium salts enter into the same class as monoamino-acids.

With the above data, the alcohol titration value of a mixture of amino-acids in solution could be calculated, even if the nitrogen distribution (Van Slyke) only were known (provided, of course, that the various amino-acids did not react together in alcohol-formalin solution, thus upsetting the actual titration values of the amino-acids collectively); *e.g.* if the distribution of nitrogen in the diamino-acids plus cystine fraction in the Van Slyke method were correct for each amino-acid, the titration value of that solution could be calculated.

WORK ON PROTEIN HYDROLYSATES.

A. *Titration values of complete hydrolysates compared with the sum of the titration values of the fractions separated by the Van Slyke method.*

Gelatin. 18 g. of pure gelatin were hydrolysed with 20 % hydrochloric acid for 36 hours and the hydrolysate evaporated twice to dryness *in vacuo*. The gummy residue was dissolved in water and decolorised with a small amount of Merck's medicinal charcoal and made up to a litre. 150 cc. were proceeded with in the ordinary Van Slyke method of separating into groups. The phosphotungstates of the diamino-acids plus cystine were decomposed by the ether-amyl alcohol method, the solution of amino-acids being freed as much as possible from hydrochloric acid and made up to 100 cc. Excess of phosphotungstic acid was removed from the filtrate by ether-amyl alcohol and the solution freed from as much hydrochloric acid as possible and made up to 250 cc. Alcohol-titration values of the original solution, filtrate, diamino-acid plus cystine fraction, and the equivalent of the amide nitrogen were determined.

Caseinogen (commercial sample 1). The same procedure as for gelatin was carried out for 18 g. of a sample of commercial caseinogen. The alcohol titration values of the original solution, the filtrate, the diamino-acid plus cystine fraction, and the equivalent of the amide nitrogen were determined.

Table I. *Gelatin and caseinogen hydrolysates. Comparison of the alcohol-titration values of the complete hydrolysates against those of the fractions separated by the Van Slyke method.*

	Gelatin	Caseinogen
Total nitrogen (g.)	0·4644	0·4245
Titration value (cc. 0·1 N alkali) of complete hydrolysate	271·8	253·9
Filtrate	218·8	172·0
Diamino-acids plus cystine ...	44·7	52·3
Equivalent of amide-N	7·5	30·4
Total of fractions	271·0	254·7
Titration equivalent as g. nitrogen	0·3794	0·3566
Nitrogen not titrated (g.)... ...	0·0850	0·0679
„ „ (% total nitrogen)	18·30	16·00

From Table I it can be seen that the sums of the titration values of the fractions were almost identical with the titration values of the original solutions. Hence, there has been no change in the titration values of the hydrolysates effected by the process of separating the fractions by the Van Slyke method.

B. *The titration values of the diamino-acid plus cystine fractions of various proteins.*

Three separate samples of caseinogen, I and II being commercial samples and III a pure sample, were investigated, each in duplicate, for distribution of nitrogen according to the Van Slyke method. The gelatin cited in A above was also studied in the same direction in duplicate (150 cc. per determination). During the course of the author's work [Davies, 1927] on the proteins of green forage plants, eight samples of proteins were prepared from green brassicaceous plants and two from umbelliferous plants and analysed by the Van Slyke method in duplicate. For each of the above proteins the different amounts of nitrogen allotted to arginine, histidine, lysine and cystine were worked out in the usual way. Part of the diamino-acid solution in each determination was used to determine the titration value in alcohol-formalin solution (after previous neutralisation of the aqueous solution to phenolphthalein).

From the amounts of nitrogen allotted in each case to the different amino-acids (not corrected for the solubilities of the bases) the theoretical titration value was calculated as cc. 0·1 *N* alkali (*i.e.* one-fourth arginine-N, one-third histidine-N, total lysine- and cystine-N ÷ ·0014). The results obtained are given in Table II.

Table II. *Calculated and actual alcohol-titration values of the diamino-acids* (*Van Slyke method*) *of various proteins.*

Source of protein	N per determination (g.)	Alcohol-titration values of diamino-acid fraction as cc. 0·1 *N* alkali		Calculated found
		Calculated	Found	
Caseinogen I	0·4245	41·36	52·30	1·27
Caseinogen II	0·4511	35·79	44·10	1·23
Caseinogen III	0·4674	47·64	57·90	1·22
Gelatin	0·4644	36·36	44·74	1·23
Cabbage I	0·4372	42·00	51·35	1·25
Kohlrabi	0·4263	41·65	49·78	1·20
Marrow stem kale leaves	0·4089	41·71	50·34	1·21
Ditto. Stems	0·4576	42·71	54·24	1·27
Turnip leaves	0·5355	53·10	63·08	1·19
Turnip root	0·4750	51·07	60·52	1·19
Cabbage II	0·4167	35·57	43·63	1·23
Turnip leaves II ...	0·3906	31·79	36·84	1·16
Carrot	0·3814	30·93	39·30	1·23
Parsnip	0·4286	42·15	50·00	1·19
				Average = 1·22

(The Van Slyke distributions of nitrogen were determined in duplicate for each of the above proteins, the average values only being used in compiling Table II.)

Experimental Note. *N*/10 aqueous alkali was used for the aqueous neutralisation and *N*/25 alcoholic potash for the alcoholic titration. 10 cc. (*ex* 100 cc. of diamino-acid solution) required from 9 to 12 cc. alcoholic potash for the titration.

C. *Titration of the monoamino-acid fraction (the filtrate of the Van Slyke method).*

The monoamino-acid fractions for most of the proteins of Table II had been treated with caustic soda and hydrochloric acid and were, therefore, unsuitable for amino-acid titration owing to separation of salts from alcoholic solution. Samples of filtrate from gelatin and caseinogen I were treated with ether-amyl alcohol to get rid of the excess phosphotungstic acid used to precipitate the bases. The solutions were evaporated to dryness twice and made up to a known volume, the titration values of aliquot portions of these being then determined (*v.* Table III).

Table III. *Titration of the specially treated monoamino-acid fractions of protein hydrolysates (obtained by the Van Slyke method).*

Protein	Total nitrogen in hydrolysate (g.)	Nitrogen in filtrate (g.)	Titration value cc. $N/10$ alkali		Calculated: found
			Calculated	Found	
Gelatin	0·4245	0·3328	237·7	226·3	0·95
Caseinogen I	0·4644	0·2608	196·3	172·0	0·92

EXPERIMENTAL NOTE. $N/10$ alcoholic potash was the strength of alkali best suited for the titration. 10 cc. (*ex* 250 cc. total solution of monamino-acids) requiring 6 to 7 cc. $N/10$ alkali for the alcoholic titration.

SIGNIFICANCE OF THE VALUES DETERMINED BY ALCOHOLIC TITRATION.

From Table I it is apparent that there has been no change in the titration values of the hydrolysates due to the processes of separating the fractions, and that losses during the operations were negligible. From Table II the titration values found for the diamino-acid solutions were uniformly greater than the expected values as calculated from the figures obtained by the Van Slyke method by 22 % of the latter.

Table III shows that the increase in titration apparent in Table II is balanced by the filtrate showing a lower titration value than that calculable for a monoamino-acid solution (a decrease of 8 % on the average). Theoretically, this solution should contain no nitrogen not accounted for by titration. As it is, the gelatin filtrate contained 0·016 g. and the caseinogen 0·020 g. not accounted for in the titration. These nitrogens, undoubtedly, belong to the bases, arginine and histidine, being the amounts not precipitated by phosphotungstic acid. Plimmer and Rosedale [1925] have advised the determination of arginine in the original hydrolysate, since its determination in the phosphotungstic precipitate gives low values. This fact is supported in this work. Van Slyke [1915] corrects for the solubility of the bases by adding 0·0032 g. nitrogen to the arginine nitrogen and 0·0038 g. to the histidine nitrogen, the total corresponding to 0·0050 g. of *untitratable* nitrogen in the filtrate. From the present work it is obvious that these corrections are too low. Plimmer and Rosedale have made similar deductions.

THE RELATION OF THE TITRATION VALUES TO THE AMINO-NITROGEN.

For the diamino-acids the titration values should be equivalent to the amino-nitrogen. That is, the equivalent of the sum of the lysine and cystine nitrogen, a third of the histidine nitrogen and a quarter of the arginine nitrogen should equal the titration value of the bases in alcoholic solution. Table II, columns 2 and 3, shows the calculated and observed values for the series of proteins. The equivalent found by titration is uniformly 22 % higher than the calculated value. Plimmer [1924] has observed the unsatisfactory results obtained by the method prescribed by Van Slyke [1915] for the determination of the amino-nitrogen of the bases. The specified time for reaction with nitrous acid is not sufficient for all the amino-nitrogen, especially that of lysine, to react and Plimmer has introduced longer periods than 5 minutes' shaking so as to get more accurate results. The results for the series of proteins (Table II) were obtained by the original Van Slyke method, and this discrepancy between calculated and observed values bears out Plimmer's observations. The uniformity of the discrepancy also seems to point out that, irrespective of the composition of the phosphotungstic precipitate, only about 82 % of the amino-nitrogen reacts during the 5 minutes' shaking. (The actual discrepancy cannot, of course, be calculated since the only basis of calculation is afforded by incorrect results.)

It is tentatively suggested, therefore, that alcohol-titration values of the phosphotungstic fraction converted into g. nitrogen give a more accurate value for the amino-nitrogen of that fraction.

In the filtrate fraction the titration value conveys nothing as to the exact state of the nitrogen in the monoamino-acids, since the nitrogen of a monoamino-acid titrates quantitatively whether it is amino- or imino-nitrogen.

OTHER SOURCES OF ERROR IN THE VAN SLYKE METHOD.

It is well known that during the Kossel and Kutscher method of determining the diamino-acids when lysine is precipitated from the arginine- and histidine-free solution, there is more nitrogen present in the phosphotungstic precipitate than can be precipitated as lysine picrate even when the picrate is crystallised out in three or four successive isolations. There is no doubt that phosphotungstic acid precipitates small but appreciable amounts of monoamino-acids under such conditions. There is the same possibility in the Van Slyke method that some of the nitrogen precipitated is monoamino-acid nitrogen. Such nitrogen titrates quantitatively in alcoholic solution and is accounted for under lysine nitrogen in the distribution table. The amount of imino-monoamino-nitrogen is comparatively small in most proteins, and its possible precipitation would account for only a small fraction of the discrepancy between the results for observed amino-nitrogen and the titration equivalent of the amino-nitrogen of the bases mentioned above. Titration values throw no light in this direction.

SUGGESTED MODIFICATIONS OF THE VAN SLYKE METHOD FOR DETERMINING THE DISTRIBUTION OF NITROGEN IN SEVEN GROUPS.

In view of the above work the following modifications of the Van Slyke method are tentatively suggested.

The complete hydrolysate. The arginine nitrogen is determined by the Plimmer method [1916] in an aliquot portion of the *original* hydrolysate, allowing for amide nitrogen driven off in the same process. The difference between the total nitrogen of the complete hydrolysate and the nitrogen equivalent to the alcohol-titration value of the same liquid is the non-titratable nitrogen and is equal to three-fourths of the arginine nitrogen plus two-thirds of the histidine nitrogen. From these two values the true arginine and histidine nitrogen values are obtained.

The phosphotungstic precipitate. The separation of the bases is carried out in the usual manner, the precipitate decomposed by the ether-amyl alcohol method and the alcohol titration value determined. The precipitated arginine and cystine are determined in the usual way, as well as the total diamino-nitrogen, so as to calculate the lysine nitrogen. The titration value converted to its equivalent weight of nitrogen is taken as the amino-nitrogen of the phosphotungstic precipitate. The precipitated histidine is calculated from the above data and the lysine determined by difference. The amounts of the precipitated arginine and histidine nitrogens are subtracted from the true values obtained from the complete hydrolysate and the differences, properly distributed into amino- and non-amino-nitrogen, allowed for in the two classes of nitrogen in the filtrate. The true arginine and histidine values are, of course, not corrected for solubility, and neither is the lysine nitrogen. Cystine nitrogen should be corrected for solubility according to the amounts laid down by Van Slyke [1912], but the amount of cystine present in ordinary proteins is too small for accurate determination by the distribution method, and no great stress can ever be put on the accuracy of this group.

The filtrate. This is best prepared for amino-nitrogen determination in the same way as the phosphotungstic precipitate, that is, the filtrate and washings from the precipitate are shaken up three times with ether-amyl alcohol mixture, the mixture being finally washed twice with dilute acid. The extracted solution is then evaporated to dryness *in vacuo* at 40°, dissolved in water and made up to 250 cc.

(Owing to nitrogenous impurities in some samples of amyl alcohol it will be found convenient to wash the half-and-half mixture of ether and amyl alcohol twice in a separating funnel with 5 % hydrochloric acid, or the mixture can be stored over 5 % acid and siphoned off as required. The nitrogenous bases, even in commercial amyl alcohol, can be completely removed by this method.)

The filtrate by this treatment can be titrated so as to check the titration value of the amino-acids precipitated by phosphotungstic acid. The quota of untitratable nitrogen in the filtrate can also be calculated and allowed for.

Practical results from the method.

Table IV sets out the results for gelatin as determined by the titration method compared with those obtained by the original Van Slyke method.

Table IV. *Results for gelatin by the titration method compared with those by the original Van Slyke method.*

	Percentage of total		
Nitrogen	Arginine	Histidine	Lysine
Titration method	17·23	2·58	5·71
Van Slyke method	14·70	4·48	6·32
	Percentage of protein		
Amino-acid	Arginine	Histidine	Lysine
Titration method	9·63	1·72	2·61
Van Slyke method	8·22	2·99	2·89
Kossel and Kutscher	9·3	0·4	2·8

For gelatin, closer values to the Kossel and Kutscher results are obtained by the modified method than by the original Van Slyke method.

SUMMARY.

Protein hydrolysates can be titrated to phenolphthalein in neutralised formalin-alcohol solution. The sum of the titration values for the amino-acid fractions separated by the Van Slyke method is equal to the titration value of the original hydrolysate.

By working in terms of nitrogen equivalent to the titratable value of solutions of amino-acid mixtures, much information can be obtained concerning the nitrogen distribution in such solutions.

The equivalent of the nitrogen of the monoamino-acid fraction should be quantitatively the same as the alcohol-titration value, but it has been found not to be completely represented by the titration value, this untitratable nitrogen representing three-quarters of the arginine and two-thirds of the histidine nitrogen not precipitated with phosphotungstic acid during the separation.

The amino-nitrogen of the diamino-acid fraction should be the equivalent of the titration value, but the titration equivalent has been found to be uniformly 22 % higher than the amino-nitrogen values determined by the nitrous acid method.

A modification of the Van Slyke method for determining the distribution of nitrogen in seven groups is suggested. This entails calculating the true values of the arginine and histidine nitrogens in the original hydrolysate, and the use of the titration value of the phosphotungstic fraction for calculating the amino-nitrogen content of that fraction.

In the practical application of the method, results more closely agreeing with the values found by the Kossel and Kutscher method are given for the diamino-acids.

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CIX. VARIATIONS IN THE p_H AND BICARBONATE OF THE PLASMA AND OF THE ALVEOLAR CO_2 DURING FORCED BREATHING.

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INTRODUCTION.

THE production of a rise in the p_H of the blood in voluntary hyperpnoea has been inferred by Davies, Haldane and Kennaway [1920] and by Collip and Backus [1920] as the result of observations made on the blood and urine during forced breathing. These observers found that the titratable acidity of the urine was decreased, and thus confirmed the experimental work of Leathes [1919].

Collip and Backus also estimated the alveolar CO_2 and the bicarbonate of the plasma, which they found were always reduced, and calculated the p_H of the blood from Hasselbalch's formula. They found that the hydrogen ion concentration of the blood was lowered.

Haldane investigated the CO_2-absorption curve of blood drawn at the end of an hour's forced breathing, and found that it was unaltered. The changes produced in the partition of the available base between the plasma and corpuscles by a lowered tension of CO_2 must therefore be transient and depend only on the alveolar CO_2 tension. He calculated that the p_H of his blood had been raised 0·38 in the course of the experiment.

Grant and Goldman [1920] using the dialysis method of Levy and Rowntree, and Peters and his colleagues [1926] using Cullen's [1922] colorimetric method, made direct observations on the p_H of the blood during hyperpnoea, and found an increase of 0·1 to 0·2 p_H above the normal.

Observations at frequent intervals have not however been carried out. The micro-method of Martin and Lepper [1926, 1] furnished us with a technique whereby we could take successive readings at intervals of a few minutes, and so study in detail the changes which take place as a result of reducing the tension of CO_2 in the alveolar air.

In some preliminary experiments on forced breathing, observations were confined to the rate of rise and the return to normal of the p_H of the blood, samples being taken at intervals of 3 minutes. Estimation of the percentage of CO_2 in the alveolar air at different rates of respiration, combined with observations on the changes which occurred in the p_H of the blood, were then made. These experiments showed us the most effective rate of respiration for the subject concerned. At the same time we found out certain precautions that had to be taken in the collection of approximately simultaneous samples of blood and of alveolar air respectively. The limits of endurance of the experimental subject (E. H. L.), who had to be responsible for the collection of blood, was also ascertained. The symptoms experienced were a feeling of chilliness and slight mental confusion, which came on within a few minutes. Increased salivation was accompanied by a troublesome cough towards the end of the experiments. Tetanic spasms were never experienced, slight rhombergism was nearly always present, and in long experiments, twitching of the muscles of the face accompanied by shakiness of the hands. During recovery the longest period of acapnoea noted was 1½ minutes. The rapid rate at which the mental symptoms came on was a constant surprise. By the end of 2 minutes it was difficult to concentrate sufficiently to count the respirations with a stop-watch. To the onlooker the effect was comparable with the early stages of alcoholic intoxication. Irritability, unreasonableness with a tendency to argue, and a lowering of the critical faculty were marked. Nevertheless, no difficulty in collecting the samples of blood was experienced so long as the routine was not departed from. The rate of respiration, once established, became almost automatic. We therefore arranged all the details of an experiment beforehand, and the subject took samples of blood only when ordered to do so and was not responsible for timekeeping. We found that we could be certain of carrying on an experiment for 25 to 30 minutes, but that after that time symptoms such as cough or shakiness of the hands might interfere with the collection of samples.

METHODS EMPLOYED.

The p_H of the blood was estimated by the Martin-Lepper [1926, 1] method. Capillary blood was collected from the finger. The saline with which the blood was diluted was adjusted to p_H 7·60 for the normal observations and to 7·75 for the forced breathing experiments.

The alveolar CO_2 was estimated with a small Haldane-Priestley apparatus. Owing to the limited time at our disposal we could only make single observations. The sample of alveolar air was always taken at the end of a forced expiration.

The bicarbonate of the plasma was estimated by the method of Van Slyke [Van Slyke, Stillman and Cullen, 1919; Van Slyke, 1922] in which excess of acid is added to a measured amount of plasma and the mixture titrated back to the discovered p_H of the blood as drawn.

The plasma was collected by pricking the ear and running the blood down a capillary tube into a glass capsule containing liquid paraffin with a small amount of solid potassium oxalate to prevent clotting. The corpuscles were spun down as soon as possible and the plasma pipetted off into a tube coated with solid paraffin. Some preliminary experiments with rabbits' blood showed us that the titration value of the bicarbonate was the same whether the cells were separated at 38° in the hot room or the blood was allowed to cool for a few minutes and then centrifuged at room temperature. We had to rely on the presence of oxalate to prevent glycolysis between the withdrawal of the blood and the separation of the cells from the plasma, it being impossible to use unneutralised solid sodium fluoride without lowering the alkali content of the blood. In view of the rapid glycolysis of blood in which the CO_2 tension has been reduced [Evans, 1922] we were doubtful as to whether the antiglycolytic action of oxalate were sufficient for our purpose, but a control experiment failed to detect any error from this cause over a period such as was used in the experiments.

The bicarbonate of the urine was estimated by Haldane's method [Davies, Haldane and Kennaway, 1920] in which excess of acid is added to 10 cc. of urine, the CO_2 shaken out and the mixture titrated to p_H 7·60.

The conditions of the experiment.

The forced breathing was carried out from ½ to 2½ hours after meals. The majority of the experiments took place about an hour after lunch. Half an hour after that meal the subject took two samples of blood for the p_H estimations and then gave a sample of alveolar air into the Haldane pipette. It is important to observe this sequence, as it is difficult when apnoeic to avoid cessation of respiration whilst blocking the mouthpiece of the sampling tube with the tongue. Blood was then collected for the bicarbonate estimation. The results of these observations gave the values for the subject when breathing naturally.

The experiment was then started. The respirations were maintained at 40 to 44 per minute, forced inspiration and expiration being carried out. In those experiments in which the p_H and bicarbonate of the plasma and the CO_2 of the alveolar air were all estimated, the following order was observed. After a period of 5 minutes' forced breathing, blood was taken for the p_H estimations, then a sample of alveolar air, then blood for bicarbonate, and further samples in the same order as soon as the gas analysis had been completed.

RESULTS.

Normal conditions.

The values obtained for p_H, alveolar CO_2 and plasma bicarbonate under normal conditions are given in Table I. p_{K_1} has been calculated from Hasselbalch's formula, after subtracting 0·22 from the p_H observed at 18° to obtain the value for the p_H of plasma at 38° [Martin and Lepper, 1926, 2]:

$$p_{K_1} = p_H^{38^\circ}\ 38^\circ \log \frac{\text{plasma bicarbonate}}{CO_2 \text{ dissolved in plasma at } 38^\circ}\ .$$

Table I.

Date (1927)	p_H (mean)	Alveolar CO_2 %	CO_2 dissolved in plasma, 38° vols. %	Plasma bicarbonate vols. % CO_2	p_{K_1}
21. iii.	7·61	6·00	3·25	51	6·195
21. iii.	7·605	6·15	3·35	48	6·229
24. iii.	7·66	5·97	3·25	54·4	6·217
24. iii.	7·68	6·03	3·3	56	6·231
24. iii.	7·678	6·03	3·3	52	6·260
26. iii.	7·667	5·90	3·2	51	6·245
26. iii.	7·678	5·85	3·17	56	6·211
7. iv.	7·65	5·95	3·23	48	6·258
10. iv.	7·607	6·05	3·3	49·75	6·209
15. iv.	7·635	5·87	3·2	45·5	6·263
24. iv.	7·624	6·11	3·35	46	6·264
25. iv.	7·629	6·04	3·3	41	6·315
					Mean = 6·241

Observations during forced breathing.

1. *The* p_H *of the blood* showed a rapid rise reaching in 10 to 15 minutes a maximum of 0·2 to 0·24 above the initial value, at which level it was maintained as long as observations could be carried out. As soon as the forced breathing was stopped the p_H fell rapidly.

2. *The percentage of alveolar* CO_2 was halved at the end of 5 minutes, this rapid fall being followed by a further gradual decrease. The return to normal is shown in Figs. 2 and 3.

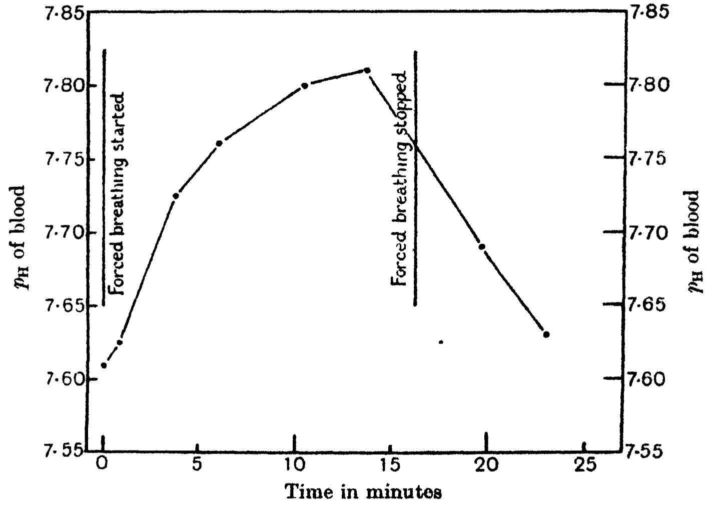

Fig. 1. Showing the rise in the p_H of plasma during forced breathing and the return to normal.

• — • — • p_H of blood.

3. *The bicarbonate of the plasma* was reduced. The titration of the plasma after forced breathing afforded results corresponding to 10 to 12 volumes % less total CO_2 than before the experiment was started. This drop was caused in two ways. The diminished CO_2 tension in the alveoli of the lungs accounts in

different experiments for 5 to 8 vols. %. The balance, 4 to 5 vols. %, is due to the higher p_H to which the plasma had to be titrated, sometimes as high as 7·85. More than the normal amount of sodium was in combination with the blood proteins, for it was found that, for the blood used, the titration value

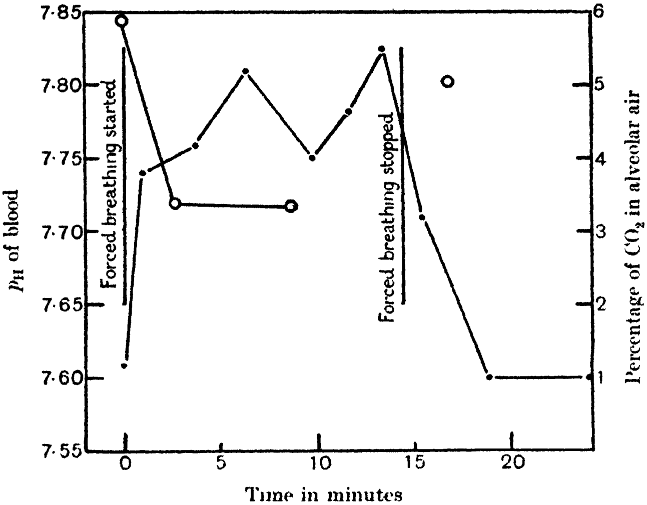

Fig. 2. Showing the rise in the p_H of the plasma and the fall in alveolar CO$_2$ during forced breathing. The somewhat low values for p_H at 4 and 10 minutes are due to the sample of alveolar air having been taken immediately before the p_H estimation.

• —— • —— • p_H of blood.

○ —— ○ - - ○ Percentage CO$_2$ in alveolar air.

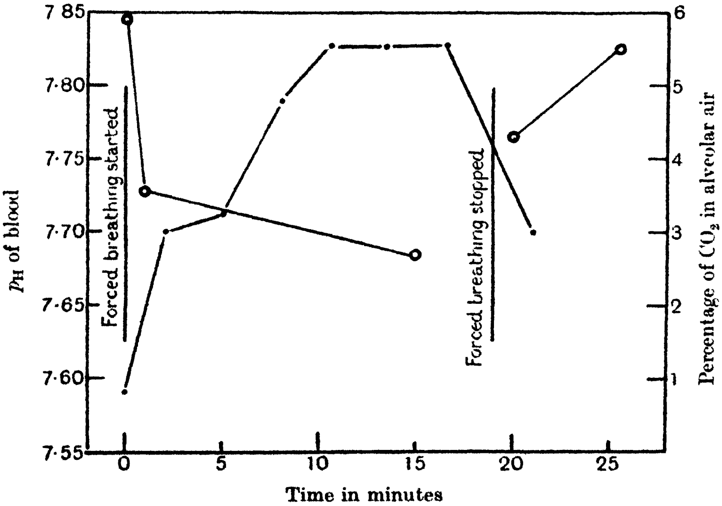

Fig. 3. Showing the rapid return to normal of the alveolar CO$_2$ when forced breathing is discontinued.

• —— • —— • p_H of blood.

○——○——○ Percentage of CO$_2$ in alveolar air.

of the plasma bicarbonate corresponded to 4 vols. % less CO_2 at p_H 7·80 than at p_H 7·60.

4. *Changes in the urine* were studied in two experiments. In both cases the urine became alkaline, containing 0·019 *M* and 0·036 *M* bicarbonate respectively. In the course of an experiment which lasted for 1 hour 0·357 g. of bicarbonate was excreted.

Fig. 4 gives a résumé of a typical experiment. It will be seen from Fig. 4 that the p_H of the blood and the alveolar CO_2 are changing continuously throughout the period of the observations. The values of p_{K_1} have therefore been calculated by interpolation from charts of the experiments, as it was impossible to estimate all three factors simultaneously. The results of a number of experiments are shown in Table II.

Table II.

Date (1927)	Duration of experiment (mins.)	p_H (mean)	Alveolar CO_2 %	CO_2, vols. % dissolved	Plasma bicarbonate vols. % CO_2	p_{K_1}
7. iv.	14	7·845	2·65	1·45	36	6·231
10. iv.	20	7·830	2·48	1·37	40	6·150
15. iv.	10	7·771	2·80	1·55	35	6·200
15. iv.	15	7·815	2·60	1·42	34·5	6·210
15. iv.	20	7·860	2·40	1·30	34	6·223
15. iv.	23	7·862	2·24	1·23	34	6·200
15. iv.	10	7·775	3·00	1·65	35	6·229
15. iv.	15	7·790	2·75	1·50	35	6·202
						Mean = 6·213

DISCUSSION OF RESULTS.

The experimental error with micro-methods is necessarily high, and when a constant depends on the value of three different micro-estimations, the errors of which may be cumulative, the results cannot agree very closely. The experimental error on the p_H observations was probably not more than ± 0·02, on the alveolar CO_2 ± 2·5 %, on the bicarbonate titration ± 1·5 %. If in any experiment a summation of errors occurred, the variation of the constant might be ± 0·08 on p_{K_1}. The mean of a number of experiments should, however, give a fair approximation to that constant. The value 6·24 obtained for p_{K_1} at 38° under normal conditions is higher than the values obtained for human blood by Warburg, 6·18, or by Parsons, 6·19 [Warburg, 1922]. These observers did not, however, add any fluoride to prevent glycolysis, and their experiments frequently lasted for 24 hours or more.

On the other hand, our bicarbonate estimations are probably slightly too low, as the solid potassium oxalate used raised the tonicity of the plasma and abstracted water from the corpuscles. The recent experiments of Eisenman [1927] would suggest that the error from this cause was not more than 1 %. Further, owing to the conditions of our experiments we could take only one sample of alveolar air, and we chose the expiratory sample.

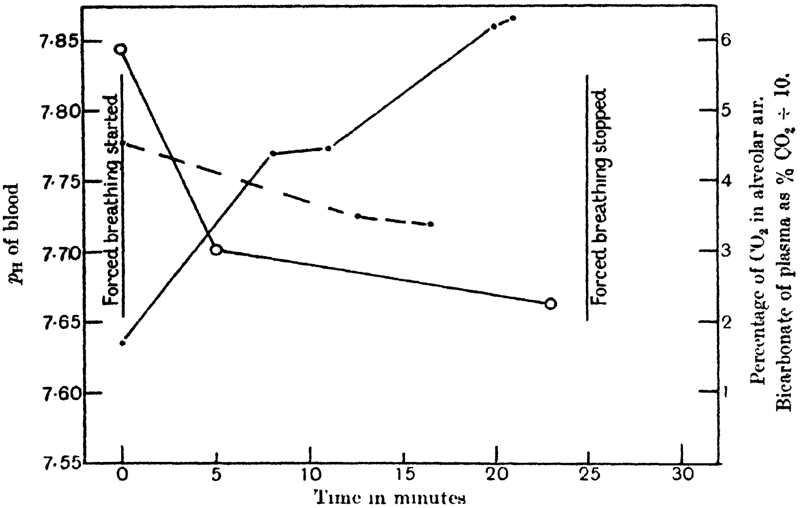

Fig. 4. The p_H and bicarbonate of the plasma and the alveolar CO_2 during forced breathing.

• — • — - • p_H of blood. O —— — O —— O Percentage of CO_2 in alveolar air.
-- — -- Bicarbonate of plasma.

Moreover, the correction factor of 0·22, which we have used for calculating the p_H at 38° from the observed value at 18° may be too low. Marrack and Thacker [1926], with a number of different bloods at the two temperatures, found a mean value of 0·247 for this correction factor.

These four considerations would all have the effect of making our value for p_{K_1} higher than that of previous observers.

The constants we obtained under normal conditions and during forced breathing are, however, comparable, and do not differ more than can be accounted for by the experimental error. We conclude therefore that in hyperpnoea carried out within 2 hours after a meal the rise in the p_H of the blood corresponds to the alteration found in the concentration of bicarbonate in the plasma and in the tension of CO_2 in the alveoli respectively.

The true nature of the "alkalosis" produced in forced breathing has been pointed out by Grant and Goldman [1920] who drew attention to the fact that, although the p_H of the blood was raised, the bicarbonate of the plasma was considerably reduced, their experiments showing an average reduction of 14 % less total CO_2. It would appear that the rise in p_H protects the body from loss of bicarbonate.

A slight definite diminution in the amount of base in the body does, however, occur during forced breathing, as evidenced by the amount of bicarbonate excreted in the urine. In Haldane's experiments this was 0·557 g., in ours 0·357 g. The loss of this quantity of alkali from the whole body could not, however, be detected by Haldane in the CO_2-absorption curve of the blood, nor by us in the titration value of the bicarbonate of the plasma.

SUMMARY.

1. Repeated observations on the hydrogen ion concentration of the blood during forced breathing have shown that the p_H rises, rapidly reaching a maximal increase of 0·20 to 0·24 in 10 to 15 minutes. When forced breathing is stopped the p_H falls even more rapidly, returning to normal within 5 minutes.

2. Simultaneous observations on the p_H and the bicarbonate of the plasma and on the alveolar CO_2 under normal conditions and during forced breathing have been made, and show that the rise in the p_H during forced breathing corresponds to the alteration found in the concentration of the bicarbonate of the plasma and the tension of CO_2 in the alveoli respectively.

We are greatly indebted to Sir C. J. Martin for much advice and helpful criticism.

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CX. THE INFLUENCE OF MEALS ON THE RISE OF THE HYDROGEN ION CONCENTRATION OF THE BLOOD DURING HYPERPNOEA.

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(*Received June 30th, 1927.*)

In the foregoing paper on the rapid rise in the p_H of the blood which occurs in forced breathing [Lepper and Martland, 1927] we confined ourselves to the discussion of experiments made not more than 2 hours after a meal. If the forced breathing was carried out 3 or 4 hours after food the course of events was always rather different, the p_H of the blood rising much more slowly and the maximum increase being never so great.

A number of experiments were made, some just before and others shortly after a meal, other conditions being as far as possible identical. The subject of the experiments (E. H. L.) had acquired considerable experience in forced breathing, and great care was taken to keep the rate and character of the respirations uniform in every case.

Fig. 1 gives the results of two typical forced breathing experiments carried out (*A*) just before, and (*B*) soon after lunch.

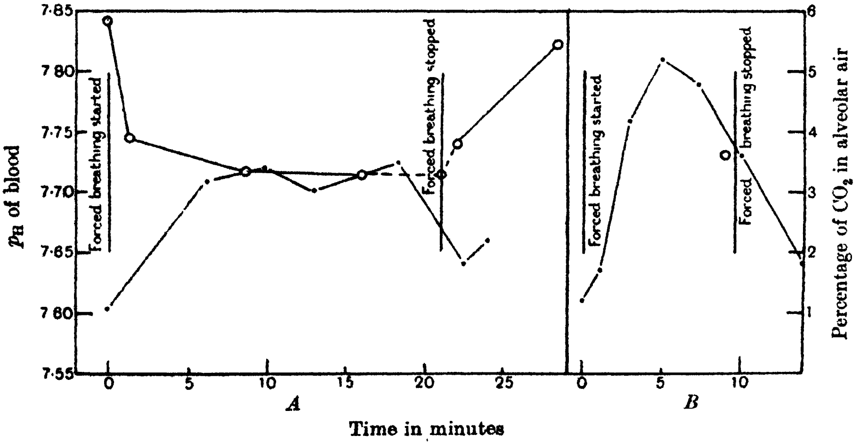

Fig. 1. The effect of forced breathing on the p_H of the blood before and after meals.

• —— • —— • —— • p_H of blood.

○ —— ○ —— ○ —— ○ Percentage of CO_2 in alveolar air.

A. Before meals. *B*. After meals.

All our comparable observations are set forth in Table I. The determinations were not always made at intervals of exactly 5, 10 or 15 minutes, but to simplify the tabular record of the results, graphs of each experiment were drawn from which values for these intervals were derived by interpolation. These are combined in Fig. 2 which shows composite curves of the observations on the p_H of the blood and the alveolar CO_2 made before and after meals. Table I shows that for equal ventilation the level of p_H reached is always lower before meals than after by not less than 0·09.

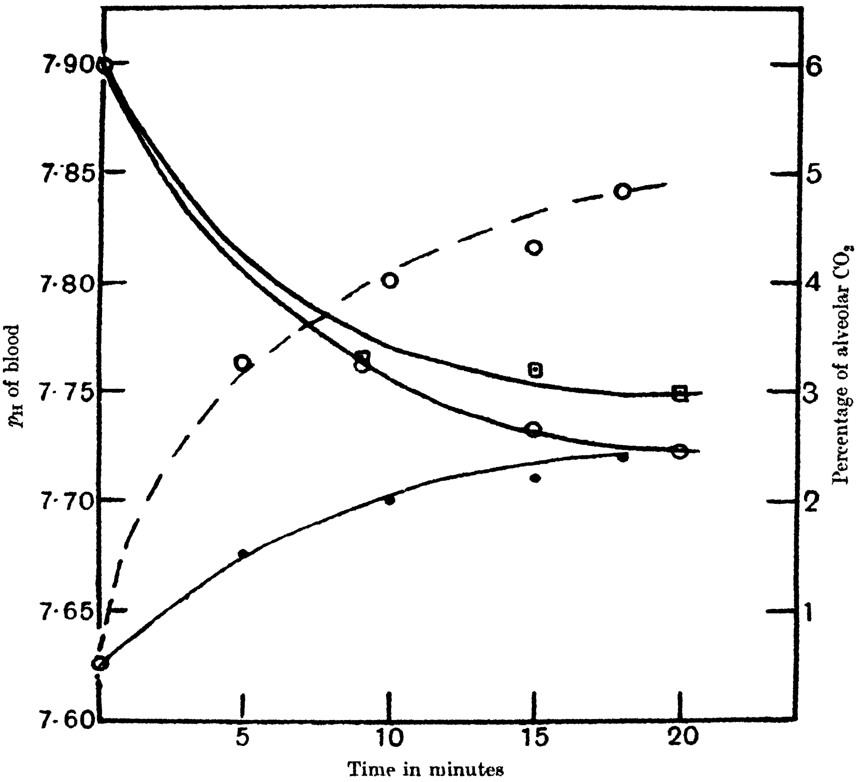

Fig. 2. Composite curves of the p_H of the blood and of the alveolar CO_2 during forced breathing experiments, carried out before and after meals

• —— • —— • —— • p_H of blood before meals.
○ — — ○ — — ○ — — ○ p_H of blood after meals.
○ —— ○ —— ○ alveolar CO_2 after meals.
▣ —— ▣ —— ▣ alveolar CO_2 before meals.

The alveolar CO_2 showed a less marked fall than in the experiments carried out soon after a meal. Notwithstanding the same respiratory efforts it was not possible to reach the same low level of CO_2 in the alveolar air before as after meals. Nevertheless, this factor is not wholly responsible for the different behaviour of the p_H of the blood. Before meals the blood appears to be more efficiently buffered. Perhaps, while gastric juice is being secreted into the stomach, hydrochloric acid escapes less readily from the corpuscles to adjust changes in the hydrogen ion concentration of the blood. This would explain why the alveolar CO_2 can be reduced to a lower level while gastric digestion is proceeding.

Table I.

		Duration of forced breathing								
Time after meal		p_H of capillary blood					Alveolar CO_2 %			
hours	mins.	0 min.	5 mins.	10 mins	15 mins.	18 mins.	0 min.	9 mins.	15 mins.	20 mins.
3	15	7·605	7·69	7·72	7·712	7·722	5·85	3·35	3·30	—
4	30	7·625	7·705	7·73	—	—	—	—	—	—
4	45	7·629	7·65	7·655	7·70	7·718	6·04	3·20	3·09	3·0
5	0	7·625	7·66	7·70	7·715	7·718	6·25	3·31	—	—
	Means	7·621	7·676	7·70	7·71	7·72	6·05	3·28	3·19	—
0	20	7·635	7·735	7·775	7·79	—	5·87	3·10	2·75	2·54
0	40	7·61	7·81	—	—	—	—	3·60	—	—
0	45	7·637	7·735	7·81	—	—	—	—	—	—
1	15	7·617	7·79	7·835	—	—	—	—	—	—
1	30	7·635	7·75	7·795	7·827	7·848	5·87	2·85	2·60	2·40
1	30	7·607	7·715	7·80	7·83	7·835	6·05	—		2·48
	Means	7·624	7·763	7·80	7·816	7·84	5·93	3·18	2·65	2·46

SUMMARY.

In forced breathing experiments carried out 3 or more hours after meals, the p_H of the blood rises more slowly, and the maximum change is less, than in similar experiments performed within 2 hours after food. Under the former conditions, the alveolar CO_2 cannot be reduced to the same extent by the same rate and depth of respiration.

We are greatly indebted to Sir C. J. Martin for much helpful advice and criticism.

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CXI. A NOTE ON THE TEMPORARY SPONTANEOUS DISAPPEARANCE OF TYPICAL "BERIBERI" SYMPTOMS IN PIGEONS FED ON DIETS DEFICIENT IN VITAMIN B.

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(*Received June 20th, 1927.*)

It has been previously reported [Kon and Drummond, 1927] that temporary improvement of the typical nervous symptoms was observed in several "beriberi" pigeons fed on a synthetic vitamin B-free diet of the type described by Randoin and Simonnet [1924]. In view of the importance of that observation it seemed necessary to repeat those experiments on a larger number of birds. Nineteen birds were actually kept under observation. The preparation of the diet and the general care of the animals were as already described [Kon and Drummond, 1927—lot 1], with the exception that the food intake was not determined; the birds were, however, weighed three times a week. The experiment lasted 54 days, during which time 4 birds (21 %), Nos. 1, 2, 5 and 7, died without showing any characteristic acute symptoms of vitamin B-deficiency. The remaining 15 (79 %) developed unmistakeable "beriberi" symptoms in the course of 24, 39, 17, 31, 34, 20, 31, 24, 28, 17, 37, 21, 47, 38 and 28 days (pigeons Nos. 3, 4, 6, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 respectively), the average period being 29 days. Of these birds 6 (32 %) (Nos. 4, 10, 11, 12, 14, 16) died without improving, whilst obvious spontaneous cures occurred in the remaining 9 (47 %), thus confirming the previous findings.

Owing to the demand on space it is not possible to give here a detailed history of each bird, nor to tabulate the results in a concise and clear table. The histories of three typical spontaneous cures are given below. Subsidence of the symptoms for a period of less than 48 hours was not considered a cure.

Pigeon No. 3. *Initial weight* 462 *g.*

Day of experiment	Weight (g.)	Temperature 0° C.	Remarks
24	305	38·5	Head retraction, convulsions
25	—	—	No symptoms
26	277	38·9	Acute symptoms provoked
27	—	39·8	Better, weak, no symptoms
28	267	41·0	" "
29	—	38·6	Looks normal, eats, but picks up food with difficulty
30	280	39·4	Crop full
31	—	38·5	Acute symptoms provoked
32	—	—	Death in convulsions

Pigeon No. 6. *Initial weight* 375 *g.*

Day of experiment	Weight (g.)	Temperature 0° C.	Remarks
17	—	37·5	Emprosthotonos, convulsions, cart-wheel turning
18	265	38·0	„ „ „
19	—	38·6	Much better
20	245	39·0	Looks quite normal, walks easily in the cage
21	—	40·2	„ „ „
22	237	38·1	„ „ „
23	—	—	„ „ „
24	222	39·8	„ „ „
25	—	—	„ „ „
26	257	40·1	Ate, can fly in the laboratory
27	—	40·1	Looks quite normal
28	228	39·9	„ „
29	—	38·0	„ „
30	209	37·4	Very marked opisthotonos, wasted
31	—	—	Found dead in the morning

Pigeon No. 9. *Initial weight* 445 *g.*

Day of experiment	Weight (g.)	Temperature 0° C.	Remarks
31	—	—	Marked head retraction, convulsions
32	266	39·0	No symptoms, better
33	—	39·0	„ „
34	247	37·8	„ „
35	—	39·7	Eats greedily
36	298	40·5	Crop full
37	—	39·5	No symptoms
38	292	—	„
39	—	38·1	Convulsions, partially paralysed
40	265	37·8	Very acute symptoms
41	—	38·5	Very acute symptoms, impaired respiration, used for another experiment in moribund condition

It was found impossible to foretell whether the symptoms would disappear spontaneously or end in the death of the animal, as sometimes even very severe initial symptoms lasting for 2–3 days eventually cleared and the birds recovered for a certain period of time (pigeons Nos. 15, 18), whereas in other cases the death of the animal suddenly followed the onset of the nervous disorder (pigeons Nos. 4, 12, 14, 16). Possibly, though not always, the fall of temperature accompanying the acute stage was more pronounced in those cases which did not recover (pigeons Nos. 11, 14, 16). It is very interesting that a second spontaneous cure was never observed, the birds either dying without marked symptoms some time after a first cure, or succumbing in a second attack, which without exception proved fatal. Two pigeons (Nos. 9 and 20) were used, after developing for the second time acute symptoms, for another experiment before death supervened; they were, however, already in a moribund condition. The second symptoms were as a rule of the most severe type, often associated with impaired respiration.

As regards the body temperature, it will be seen that in general the onset of acute symptoms is accompanied by a marked drop and that there is a corresponding increase when a spontaneous cure is manifested. There are, however, a few exceptions, *e.g.* pigeon 17. The body temperature of a "deficient" bird is the resultant of so many factors: inanition, impaired temperature regulation, daily fluctuations and last, but not least, infection [McCarrison, 1921], that it is almost impossible to account satisfactorily for all of them and gain a clear picture of the true condition of the animal.

In order to be sure that the spontaneous cures were not caused by an unknown disturbing factor present in the laboratory, a batch of 24 pigeons purchased at the same time and from the same source as the first 19 birds, was placed under observation simultaneously with them and under identical conditions except that their diet consisted of polished rice instead of the synthetic mixture. Those birds behaved absolutely normally: a large percentage (over 60 %) developed typical symptoms, but not a single spontaneous cure could be observed, the animals dying mostly in the course of 24, maximally 48, hours after the onset of the nervous disorder.

Whether the known deficiencies of polished rice—insufficient amount of salts and lack of roughage—are to be looked upon as the causative agents of the onset of immediately fatal "beriberi" symptoms or whether still another factor plays here a rôle can be decided only on the results of further investigations.

These experiments clearly demonstrate that a curative test, when pigeons fed on synthetic rations of the type here described are used, might lead to completely erroneous conclusions.

SUMMARY.

The occurrence of temporary spontaneous cures in a large percentage of pigeons fed on a vitamin B-deficient but otherwise complete synthetic ration of the type described by Randoin and Simonnet reported in a previous communication of Kon and Drummond is fully confirmed. Pigeons fed on synthetic rations should not be used for the curative test.

I wish to take this opportunity of expressing my deep gratitude to Prof. J. C. Drummond for the hospitality of his laboratory and for other facilities which he so kindly extended to me, and also to thank the International Health Board of the Rockefeller Foundation for a Fellowship, during the tenure of which this work was carried out. The expenses were defrayed from a grant made by the Medical Research Council.

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CXII. THE EFFECT OF THE ADMINISTRATION OF GLYCINE TO PIGEONS ON A DIET DEFICIENT IN VITAMIN B.

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(*Received June 23rd, 1927.*)

It is believed by various authorities [see Funk, 1914; Funk and Collazo, 1925; Randoin and Simonnet, 1924, 1, 2] that protein metabolism requires less vitamin B than that of carbohydrate. In fact these authors consider vitamin B as specially associated with the utilisation of carbohydrates, the last-named authors even believing that experimental animals (*e.g.* pigeons and rats) can be kept alive in a good state of nutrition on dietaries deprived both of vitamin B and of carbohydrates. Such theories have been fully dealt with in a previous communication [Kon and Drummond, 1927], in which observations were reported in disagreement with them. It seemed worth while, however, to ascertain whether the alleged sparing action attributed to proteins could be explained by reference to their specific dynamic action, leading to increased cell metabolism and possibly to liberation of the body reserves of vitamin B. In order to investigate this question, it was decided to administer to pigeons fed on a diet deficient in vitamin B sufficient amounts of glycine to raise markedly their metabolism. Three lots of pigeons, each consisting of four birds, were used. The initial average weights were 428, 421 and 411 g. respectively. At first they were fed on polished rice for 12 days until the typical decrease in appetite was manifested, by which time the average weights were 393, 388 and 362 g. respectively. The actual experiment was then started, the first lot receiving daily a solution of 2 g. of pure synthetic glycine in 25 cc. of water, the second a solution of 1·6 g. of glucose in 12 cc. of water (glycine gives rise to 80 % of its weight of glucose [Ringer and Lusk, 1910]), the third 25 cc. of water. Ample supplies of polished rice and water were also provided for each group. All the liquids were introduced into the crop by means of a small funnel and a piece of rubber tubing. The weight of the birds, their food intake and cloacal temperature were determined daily. The glycine and glucose solutions were diluted as far as convenient for administration in order to avoid the introduction of strongly hypertonic fluids. Nevertheless, the glucose solution had roughly 2·4 times the osmotic pressure of blood, and the glycine 3·3. Vomiting was observed in all the pigeons of the glycine group. It was not present in the two remaining sets. Vomiting was frequently observed by Csonka [1915] after administering glycine to dogs.

Effects of dosage. The birds receiving glycine died in the course of 30, 24, 21 and 25 days respectively (on the 18th, 12th, 9th and 13th days after the administration of glycine was commenced) without showing any characteristic symptoms other than loss of appetite, weakness and in two cases paralysis. Nothing of interest was revealed on *post mortem* examination with the exception of a marked enteritis in one case (pigeon 3). One of the pigeons receiving sugar developed opisthotonos on the 28th day of the experiment, one died without marked symptoms on the 27th day. The experiment was terminated on the 31st day, after all the pigeons receiving glycine had died. Two of the sugar group and all the birds of the water group were still surviving at that time.

Body weight. After 8 days of actual experiment (up to the death of the first pigeon of the glycine group) the average weights were 339, 339 and 321 g. respectively, the pigeons on glycine having lost 54 g., or an average of 6·8 g. daily, those on sugar 49 g., or an average of 6·1 g. per day, and the birds receiving water 41 g., or 5·1 g. daily.

Food intake. The average daily food intake, that is, the voluntary consumption of polished rice, for the same period of time was 0·5 g. in the glycine group, 1·6 g. in the sugar group and 4·2 g. in the water group. It appeared that administration of glycine and glucose interfered with the voluntary food intake, in the former case more strongly than the latter.

Body temperature. The average temperatures at the beginning of the actual experimental period were 41·7°, 41·9° and 41·8°; at the end of the experiment these had dropped to 39·6°, 40·7° and 40·4°, the heaviest drop being manifested by the glycine group.

It is evident that the administration of a substance having a marked specific dynamic action had no beneficial effect on pigeons deprived of vitamin B and that on the contrary these birds declined rapidly. There is little doubt that glycine had no toxic action *per se*, and that probably the bad effects were caused by the high molecular concentration of the liquid administered. The molecular concentration of the glucose was lower and hence the untoward results were much less marked. It will be remembered that in pigeons forcibly fed deficient diets, the crop does not function normally [Kon and Drummond, 1927], and that the hypertonic fluid consequently could not be disposed of so easily as in normal animals.

Funk and Paton [1922] observed occlusion of the crop and death in pigeons fed forcibly a mixture of glucose and egg-albumin, and attributed these symptoms to a difference in osmotic pressure of the sugar solution and the body fluid. Collazo [1923] introduced large amounts (10 g.) of various carbohydrates into the crops of vitamin B-deficient and normal pigeons and found that beriberi symptoms and death, or death without previous symptoms, ensued invariably in the former. Glucose, galactose and fructose showed the highest, and starch the lowest toxicity, whereas the disaccharides sucrose, lactose and maltose, occupied an intermediate position. No symptoms were produced in normal animals even by larger doses. Collazo [1923] concludes from his experiments that in vitamin B avitaminosis, the carbohydrate metabolism becomes aberrant and toxic intermediate substances are produced. It seems that a much simpler explanation is that, the emptying of the crop being impaired in vitamin B-deficient pigeons, they were killed by the enormous osmotic pressure of the solutions. Collazo does not give the concentration but it was surely not less than 20 % as even the administration at once of 50 cc. of liquid would present difficulties[1]. In normal pigeons the rapid emptying of the crop not only exposes the hypertonic liquid to the large surface of the gut and thus ensures prompt dilution and excretion, but also enables the birds to drink water. The supposition is further strengthened by the fact that the toxicity of the substances administered was inversely proportional to their molecular weight (starch, disaccharides, monosaccharides). It is to be noted that in the dog administration of glucose (100 g.) never caused death, but vomiting, a symptom observed by Cowgill [1921] even when vitamin B-containing extracts were given by the stomach tube to vitamin B-deficient dogs. Collazo also found that administration of 1 g. glucose daily to a pigeon, previously kept for 1 week on a vitamin B-deficient ration, caused convulsions and death in the course of 3 days. As already reported, out of four pigeons receiving nearly twice this dose, two died 12 and 16 days respectively after the administration was commenced. Two were still surviving after 31 days of vitamin B-free feeding and 19 days of sugar administration.

SUMMARY.

Administration of glycine has no beneficial effects on pigeons fed on diets deficient in vitamin B. The opinion of Collazo that the carbohydrates administered to vitamin B-deficient pigeons are toxic *per se* is not corroborated. The effects are attributed to the high osmotic pressure of the solutions administered and to the inability of the deficient birds to empty their crops normally.

My best thanks are due to Prof. J. C. Drummond for his interest and support during the course of the work and also to the International Health Board of the Rockefeller Foundation for a Fellowship, during the tenure of which this work was carried out.

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[1] It would appear from a subsequent publication of Rubino and Collazo [1923] that the carbohydrates were administered as 50 % solutions.

CXIII. THE EFFECT OF CYANIDE ON THE SCHARDINGER ENZYME.

By MALCOLM DIXON (1851 *Exhibition Senior Student*).

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(*Received June 25th, 1927.*)

Szent-Györgyi [1926] claims to have brought forward evidence that in the aerobic oxidation of aldehydes under the influence of the aldehyde oxidase of milk (Schardinger's enzyme) an activation of the molecular oxygen by iron in accordance with Warburg's theory is necessary. His evidence consists in the observation that cyanide in a concentration of $M/200$ produced a 75 % inhibition of the uptake of oxygen by the enzyme-aldehyde system, and he concludes that the cyanide acts by inactivating the iron, as it does in the cases of iron catalysis studied by Warburg and others. The question is of importance in connection with Warburg's theory of tissue oxidations, and in view of the facts that Dixon and Thurlow [1925] have shown that the xanthine oxidase system is independent of such activation by iron, and have further shown [1924, 2] that the xanthine oxidase is identical with, or at any rate very closely similar to, the Schardinger enzyme, it was clear that a more detailed study of the action of cyanides on the latter enzyme should be made.

The experiments were carried out in a Barcroft apparatus at 40°. The enzyme solution used was a 20 % solution in phosphate buffer of the caseinogen preparation of Dixon and Thurlow [1924, 1]. This preparation is very similar to that used by Szent-Györgyi, differing only in the method of precipitation. The acetaldehyde was freshly prepared from pure crystalline aldehyde-ammonia by distillation with dilute sulphuric acid. It was made up in solution in water, and at Dr Szent-Györgyi's suggestion the solution was made alkaline to phenolphthalein with NaOH, allowed to stand a few moments, and re-neutralised immediately before use in order to destroy any traces of peracids which might have been formed and which might interfere with the action of the cyanide. The same amount of aldehyde solution was placed in both flasks of the apparatus in order to compensate the vapour pressure. The cyanide solution was always a freshly made up and neutralised solution of pure crystalline potassium cyanide.

Several series of experiments were carried out, and similar results were obtained in all. A typical set of curves is given in Fig. 1.

It will be seen that $M/100$ cyanide produces some inhibition of the oxygen uptake, but much less than that observed by Szent-Györgyi with $M/200$, possibly owing to slight differences in the experimental conditions. With *M*/1000 cyanide, however, no inhibition is produced, which shows that the system is *not* cyanide-sensitive in Warburg's sense. Even with *M*/300 cyanide no effect can be observed, as Fig. 2 shows, and the inhibition only begins to be noticeable with about *M*/200.

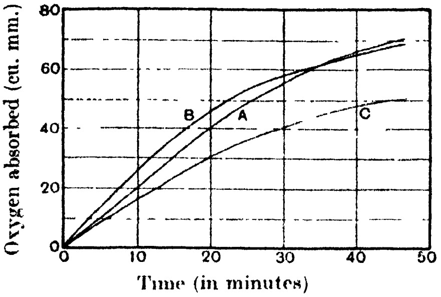

Fig. 1.

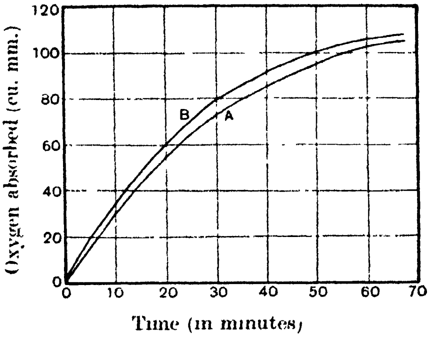

Fig. 2.

Fig 1. Each apparatus contained: 1 cc. 20 % caseinogen enzyme preparation; 1 cc. phosphate buffer (p_H 7·6): 1 cc. 0·5 % acetaldehyde; and the following concentrations of KCN: *A*, 0; *B*, *M*/1000; *C*, *M*/100. The control flasks contained the same solutions, but with buffer instead of the enzyme solution.

Fig. 2. As in the previous experiment, but a different enzyme preparation was used, and the concentrations of KCN were: *A*, 0; *B*, *M*/300.

We may compare the behaviour of this system with that of a typical cyanide-sensitive system—the succinoxidase of muscle, which was studied by Fleisch [1924]. Fleisch showed that in this case a concentration of cyanide of less than *M*/1000 was amply sufficient to produce a practically complete inhibition of the oxygen uptake in the presence of succinate. The enzyme was not, however, destroyed by the cyanide, as the methylene blue reduction was unaffected.

In order to determine whether the inhibiting action of *M*/100 cyanide on the Schardinger enzyme was due to actual destruction of the enzyme, the solutions used in the experiment given in Fig. 1 were transferred at the conclusion of the experiment from the flasks of the Barcroft apparatus to Thunberg vacuum-tubes. 1 cc. of 1/5000 methylene blue was added to each tube and the tubes were then evacuated and placed in the thermostat at 32°. There was no need to add fresh aldehyde, as only about 10 % of that originally added had been oxidised during the course of the experiment. The results obtained were as follows:

Contents of tube	Reduction-time min.
Solution from apparatus with no KCN...	98
" " *M*/1000 KCN	90
" " *M*/100 KCN	2000
1 cc. original solution + 1·1 cc. buffer + 0·9 cc. aldehyde	9

These figures show quite definitely that the inhibiting effect of the higher concentration of cyanide is due to an actual destruction of the enzyme itself. In all three solutions there had been considerable destruction during the oxygen uptake, which was doubtless due to the peroxide formed during the reaction, as already observed during the oxidation of hypoxanthine [Dixon, 1925; see also Thurlow, 1925]. The much greater destructive effect of the *M*/100 cyanide is however evident.

A precisely similar inhibitory effect of cyanide in concentrations greater than about *M*/150 was observed by Dixon and Thurlow [1925] using hypoxanthine as substrate, and shown by them to be due to the same cause.

Some unpublished experiments made by the writer and Miss Thurlow in 1924 may be given here, as they show clearly the destructive effect of cyanide on the milk oxidase, with either aldehyde or hypoxanthine as substrate. The experiments were carried out in Thunberg tubes in the usual way. The enzyme solution used was one of the caseinogen enzyme preparation in phosphate buffer at p_H 7·6.

Contents of tubes	Reduction-time min.
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. (0·2 mg.) hypoxanthine	5
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. (0·2 mg.) hypoxanthine + 0·1 cc. *M*/10 KCN	4·5
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. (0·2 mg.) hypoxanthine + 0·5 cc. *M*/10 KCN	7
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. 10 % aldehyde	7
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. 10 % aldehyde + 0·1 cc. *M*/10 KCN	8·5
5 cc. enzyme + 0·5 cc. methylene blue + 0·1 cc. 10 % aldehyde + 0·5 cc. *M*/10 KCN	17

These figures show that while *M*/500 cyanide produces no appreciable effect, *M*/100 cyanide has a definite destructive action on the enzyme; which is in good agreement with the experiments given above. The effect appears to be more marked with aldehyde than with hypoxanthine, but this does not necessarily mean that we are dealing with two distinct enzymes, as it is possible that there may also be an action of the cyanide on the aldehyde at the enzyme surface.

The destruction may become very marked if the oxidase is allowed to stand for a short time with the cyanide before adding the aldehyde and methylene blue, as the following experiment shows. 1 cc. of 20 % caseinogen preparation + 1 cc. *M*/30 KCN were allowed to stand 5 minutes at room temperature, and then 1 cc. 1 % aldehyde + 1 cc. buffer and 1 cc. methylene blue added. The reduction-time was 30 minutes, whereas 1 cc. of the same enzyme solution + 1 cc. buffer + 1 cc. aldehyde + 1 cc. methylene blue reduced in 4½ minutes.

The experiments described in this paper show clearly that the inhibition produced by the higher concentrations of cyanide cannot be regarded as evidence for oxygen-activation. Since it occurs with methylene blue as the hydrogen acceptor under precisely the same conditions as with oxygen it must be referred rather to an actual destruction of the enzyme.

SUMMARY.

Inhibition of the aerobic oxidation of aldehyde by the Schardinger enzyme is produced only by concentrations of cyanide greater than *M*/200. It is due to an actual destruction of the enzyme itself, and does not constitute evidence of oxygen-activation.

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CXIV. ON THE REDUCING POWER OF GLUTATHIONE AND CYSTEINE.

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(*Received June 25th, 1927.*)

Kendall and Nord [1926] in a recent paper have put forward a somewhat novel view of the mechanism of reduction by —SH groups. They state that, in the absence of oxygen, cysteine and reduced glutathione are unable to reduce indigo carmine, but that in the presence of small amounts of oxygen an active addition compound of the —SH group with oxygen is formed, whereby the system acquires the power to reduce the dye. The same "activation" was obtained by the addition of hydrogen peroxide or of sodium disulphide.

In view of the importance of such a fact in considering the functions of sulphur compounds in cell respiration processes, the experimental basis for such a view calls for careful consideration.

THE REDUCING POWER OF CYSTEINE AND GLUTATHIONE IN THE ABSENCE OF OXYGEN.

Kendall and Nord placed solutions of indigo carmine and cysteine in phosphate buffer in a vessel through which nitrogen was bubbled continuously. After 40 hours at 30° the solutions still remained deep blue, from which they concluded that no reduction of the dye had taken place. This result was at variance with some unpublished observations made by us in 1922, in which, using the Thunberg vacuum-tube technique, rapid reduction of indigo carmine by cysteine was found to occur. It seemed to us that the discrepancy might be due to the presence of traces of oxygen, particularly in view of experience of the difficulty in obtaining nitrogen in quantity free from such traces.

In view of the large volume of gas which Kendall and Nord must have passed through their solution it will be clear that a very small percentage of oxygen in the nitrogen might have been sufficient to prevent decoloration, owing to the great ease with which reduced indigo carmine is reoxidised by oxygen. Assuming (information on this point being absent from Kendall and Nord's paper) a rate of bubbling of 1 cc. per second, then in 40 hours 144 litres of nitrogen would flow. Even if the gas were 99·99 % pure this would mean that nearly 15 cc. of oxygen would pass into the solution during the experiment, a quantity sufficient to reoxidise the whole of the indigo carmine many times over.

In order to decide whether cysteine can reduce indigo carmine in the absence of oxygen we made experiments, taking extreme precautions to remove all traces of oxygen. For this purpose the special vacuum-tube represented in Fig. 1 in front and side view was employed.

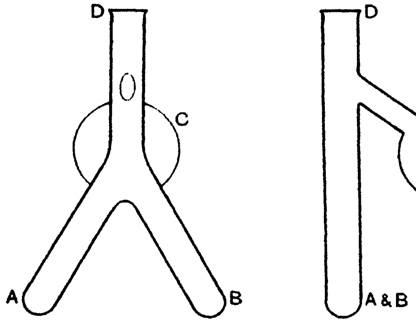

Fig. 1.

The open end *D* was provided with a well-fitting rubber stopper carrying a glass tube with a tap, so that the tube could be evacuated. The neutral cysteine solution (previously kept in an exhausted vacuum-tube for 2 or 3 days) was placed with an appropriate volume of buffer solution (p_H 7·6) in the limb *A*, and the indigo carmine solution in *B*. A strong solution of sodium hydrosulphite in water and caustic soda were placed in the bulb *C* by means of a special curved pipette, and the apparatus immediately exhausted by an efficient water-pump. When no further bubbles of dissolved gas could be obtained from the liquids, nitrogen was admitted and the apparatus re-exhausted. The process was again repeated, the tap closed after the final exhaustion and the apparatus allowed to stand for 24 hours or more with gentle shaking at intervals. At the end of this period the solutions in *A* and *B* were carefully mixed by tilting the tube, and the apparatus was placed in the bath at 40°. (The apparatus was so constructed and handled that there was no possibility of any trace of the hydrosulphite getting into the reacting solutions. In no case have we observed the slightest reduction of the dye before the solutions were mixed, or in control experiments without the —SH compound.) In all cases rapid and complete reduction of the indigo carmine occurred. Using 0·3 cc. indigo carmine (0·035 *M*) + 1·0 cc. cysteine (0·05 *M*) + 3 cc. phosphate buffer the dye was completely reduced in 6 minutes. At the end of the experiment the vacuum in the tube was tested by immersing the tube in water and opening the tap. The water filled the tube with the exception of a small bubble of approximately 50 mm.^{3}. Assuming that our nitrogen contained as much as 1 % of oxygen it will be clear that the maximum possible quantity of oxygen in the tube at the end of the final exhaustion is 0·5 mm.^{3}, and this will of course be rapidly and completely absorbed by the hydrosulphite solution, which as is well known is an extremely efficient deoxygenating agent.

In a series of experiments we found that the more carefully traces of oxygen are removed, the smaller becomes the time for reduction of the dye (provided that large amounts of oxygen are not present; cf. the following section).

Similar results have been obtained with reduced glutathione.

The preparations of cysteine used by us, though very pure, were not absolutely iron-free in Warburg's sense, although the fact that no trace of colour developed in the solutions on making faintly alkaline with ammonia and shaking with oxygen shows that this was very nearly the case. Kendall and Nord freed their cysteine preparations from iron by the Warburg and Sakuma [1923] technique; but the effect of this was largely negatived by the fact that they used, without purification, commercial indigo carmine, which would certainly contain small amounts of iron. In view of the recent work of Harrison [1927], who found that the reduction of methylene blue by cysteine was largely (but not entirely) due to catalysis by traces of iron, it became necessary to consider how far the reduction of indigo carmine observed by us was due to the same cause. We obtained with indigo carmine results very similar to those obtained by Harrison with methylene blue. The addition of very small traces of ferric chloride produced a marked acceleration of the reduction, while the addition of a small amount of KCN slowed the reaction considerably. But, as is the case with methylene blue, even when the metal-catalysis is completely abolished by the addition of KCN, and when all traces of oxygen are removed, the reaction still proceeds with a quite considerable velocity.

We consider that these results dispose of Kendall and Nord's contention that cysteine and glutathione cannot reduce indigo carmine in the absence of oxygen. As this contention is fundamental to their theory, we propose to discuss the bearing of the facts given above on their evidence for the formation of an oxygen addition compound.

When Kendall and Nord stopped the flow of gas through their solutions, and allowed them to stand undisturbed for some hours, they observed that occasionally the dye became reduced, with the exception of a blue layer on the surface of the liquid. Kendall and Nord concluded that in these cases oxygen had leaked into the apparatus, and by its presence had enabled the reduction to be brought about, owing to some activating property. In order to test this hypothesis they proceeded as follows. After bubbling nitrogen through the mixture of cysteine and indigo carmine for several hours, with no apparent reduction of the dye, the bubbling was stopped and 10 cc. of air admitted. On allowing to stand undisturbed for 2 hours, reduction was complete in a layer 2 cm. below the surface.

A possible interpretation of this experiment seems to be that the traces of oxygen continuously introduced by the nitrogen maintained the dye in its oxidised form. On cessation of bubbling the rate of diffusion of oxygen into the deeper layers of the solution is not sufficient to mask the effect of the reduction by the —SH compound. There therefore results a colourless deep layer and a blue surface layer as Kendall and Nord observed.

This conclusion is strikingly supported by the following internal evidence in Kendall and Nord's account.

(1) "The reduction was retarded in proportion to the agitation," *i.e.* to the volume of gas passing through the solution.

(2) "When the air was mixed with the nitrogen and bubbled through the solution, little or no reduction of the indigo carmine occurred"; this is a very significant statement, since there seems no reason on their view why oxygen leaking into the apparatus should be able to cause the formation of the addition product, while oxygen deliberately introduced with the nitrogen should be unable to act. In order to account for this result Kendall and Nord were obliged to assume that agitation prevents the formation of the oxygen addition product!

In view of the above facts, which would appear to invalidate Kendall and Nord's experiments, we considered it necessary to reinvestigate the question whether oxygen can *accelerate* the reduction of indigo carmine. In the presence of fairly large amounts of oxygen we have been able to observe such an acceleration. For instance, if a neutral solution containing cysteine and indigo carmine is divided into two portions, one being placed in an evacuated Thunberg tube and the other in an open test-tube, it will be found that reduction is complete in the open tube (except of course at the surface of the liquid) definitely sooner than in the vacuum-tube.

We are of the opinion that this phenomenon is due to the hydrogen peroxide formed during the autoxidation of the —SH group [Thurlow, 1925], and we discuss its interpretation more fully in the following section of the paper.

THE RÔLE OF HYDROGEN PEROXIDE IN THE FORMATION OF THE ADDITION COMPOUND.

Kendall and Nord state that hydrogen dioxide is also able to form an active addition compound: "hydrogen dioxide was therefore added to solutions of cysteine and after some time indigo carmine added. No reduction occurred. The indigo carmine was then added to the cysteine solution before the hydrogen dioxide. With the conditions thus produced the indigo carmine was reduced almost immediately providing sufficient hydrogen dioxide had been used." Kendall and Nord consider that these results indicate the existence of an essential intermediate compound which is formed by the action of hydrogen dioxide on cysteine.

We have confirmed these observations, but we do not agree with this interpretation of the phenomenon. It is obvious that the action of the hydrogen peroxide may be either on the cysteine or on the indigo carmine. The fact that according to Kendall and Nord the oxygen addition compound is only formed when indigo carmine is present, for which they offer no satisfactory explanation, suggests that the active compound may be due to the action of the peroxide on the dye rather than on the cysteine. It is well known that hydrogen peroxide reacts readily with indigo carmine, and with dyes on which hydrogen peroxide has no action Kendall and Nord failed to obtain effects comparable with those observed with indigo carmine. We have found for instance that hydrogen peroxide produces no acceleration of the reduction of methylene blue by cysteine.

In order to test the possibility that a catalytically active compound is formed from the indigo carmine we carried out the following experiments.

A solution of indigo carmine in phosphate buffer p_H 7·6 was treated with dilute hydrogen peroxide, and the mixture allowed to react until *slight* bleaching had occurred. Manganese dioxide was then added, the solution warmed to decompose all traces of H_2O_2, and the mixture filtered (solution *A*). A second solution of untreated indigo carmine was then prepared (solution *B*) of the same depth of colour as solution *A*. The two solutions were thus identical, except that *A* contained a small amount of the oxidation-product of indigo carmine. Equal volumes of the two solutions were placed separately in two Thunberg tubes, equal amounts of cysteine solution added to each, and both tubes thoroughly evacuated. It was found that the treated indigo carmine solution (solution *A*) was reduced with very great rapidity compared with solution *B*, the acceleration produced being comparable with that produced by the direct addition of hydrogen peroxide. That a true reduction, and not merely a bleaching, had occurred was shown by the ready reappearance of the colour on the admission of oxygen. A control was carried out, to make certain that all traces of peroxide had been removed, by adding manganese dioxide to dilute hydrogen peroxide of the same strength as previously and treating in exactly the same way as before. The filtrate gave no test with KI and starch, and on addition to a mixture of indigo carmine and cysteine no accelerating effect could be observed. A further control, in which indigo carmine was treated with MnO_2 without H_2O_2, gave no acceleration; showing that the acceleration previously observed was not due to catalytic action by traces of manganese.

The result described shows conclusively that by the action of hydrogen peroxide on indigo carmine products are formed which have a marked catalytic effect on the reduction of indigo carmine by cysteine. We consider that this fact provides the true explanation of the phenomena observed by Kendall and Nord, and renders unnecessary the assumption of the formation of an active oxygen addition product with cysteine.

The fact that no effect was observed by Kendall and Nord when the hydrogen peroxide was added to the cysteine, and indigo carmine added only after some time, is clearly due to the removal of the H_2O_2 by the usual reaction with the —SH group before the addition of the indigo carmine. By reduction-potential measurements we have found that under the above conditions, removal of the peroxide is complete in a few minutes.

Hydrogen peroxide (as is so frequently the case) probably requires traces of iron as a catalyst for its action on the indigo carmine. We have found that minute traces of ferric or ferrous salts have a marked accelerating effect on the bleaching of indigo carmine by H_2O_2; and we have shown that the products of the reaction are active accelerators. Moreover, we have found that cyanide definitely inhibits this reaction. Now if our interpretation be the correct one we should expect to find that the addition of a small amount of KCN, by preventing the action of the peroxide on the dye, would abolish the accelerating effect of the H_2O_2. This is precisely what occurs, as the following experiment shows.

Contents of tubes	Reduction-time at 35°
Indigo carmine + buffer + cysteine	1 hr.
,, + ,, + ,, + H_2O_2	17 mins.
,, + ,, + ,, + ,, + KCN	3 hrs.
,, + ,, + ,, + KCN	4 hrs.

The quantities used were:

Indigo carmine: 1 cc. of 0·035 *M*.
Cysteine: 1·5 cc. of 0·1 *M*.
H_2O_2: 0·5 cc. of 0·1 *M*.
KCN: final concentration 0·005 *M*.
Phosphate buffer: 3 cc. $p_H = 7·6$.

It will be seen that the presence of the cyanide completely prevents the acceleration by the peroxide, although the normal reduction of the indigo carmine by the cysteine still occurs, as mentioned above.

We conclude from these facts that Kendall and Nord's main phenomenon is dependent on the presence of traces of iron present as impurities, perhaps as simple iron salts, perhaps as a cysteine-iron complex.

THE RÔLE OF SODIUM DISULPHIDE IN THE FORMATION OF THE ADDITION COMPOUND.

Kendall and Nord state that addition of 0·01 *N* sodium disulphide solution to cysteine in the presence of indigo carmine brought about the prompt reduction of the dye, and they claim that "cysteine reacts with sodium disulphide in the same manner as it does with hydrogen dioxide."

No information is given as to the method of preparation of the disulphide solution. As ordinarily prepared, by dissolving the theoretical amount of sulphur in a solution of Na_2S, the solution is a most powerful reducing agent, and we have found that it reduces indigo carmine with remarkable rapidity. Kendall and Nord, in fact, also observed this reducing power.

It therefore seems, to say the least, inadvisable to draw such a definite conclusion as that of Kendall and Nord, since the reduction of the dye may be due to the sodium disulphide and not to the cysteine. Kendall and Nord do not appear to have carried out control experiments with sodium disulphide alone.

REDUCTION-POTENTIAL MEASUREMENTS.

Dixon and Quastel [1923] pointed out the unsuitability of the platinum electrode for work on the —SH system, owing to the extensive potential drifts which are met with using this electrode. Kendall and Nord nevertheless carried out all their work with this electrode. They "obtained systems in which the drifts had been eliminated" by simply waiting until the drifts had ceased, and taking the final reading. The significance of these values seems somewhat doubtful.

Furthermore, the hydrogen ion concentrations of Kendall and Nord's solutions were not accurately controlled, and moreover were undetermined. The additions made at various times to their electrode vessels consisted of such solutions as those of cysteine hydrochloride, the sodium salt of cystine, sodium disulphide, and un-neutralised glutathione, together with "sufficient $N/10$ sodium hydroxide to neutralise *all or part* of the acidity" (our italics).

Their method of preparation of solutions of reduced indigo, which they used in experiments on the reversibility of the —SH, —SS— system, seems also to call for some notice [see Kendall and Ort, 1926]. The indigo was reduced by boiling with glucose and sodium hydroxide; and the whole of the resulting mixture of reduced indigo, alkali, and the decomposition products of glucose was used for addition to the electrode vessel, and simply referred to as "reduced indigo" by Kendall and Nord. It will be obvious that such a solution, containing one of the most powerful reducing agents—glucose in the presence of alkali—together with the unknown decomposition products of glucose, will give reduction-potential readings of little significance.

Kendall and Nord state that neutralisation of the above solution was unnecessary, as on oxidising the reduced indigo and filtering off the indigo the resulting solution was neutral to phenol red. We have found, however, that this indicator cannot be used to determine the p_H of such a solution, as on addition of phenol red to a solution of glucose and NaOH which has been boiled, the indicator always assumes a colour approximately corresponding to p_H 7·2. Addition of 40 % caustic soda fails to alter this colour, so that this indicator gives no information as to the p_H of such solutions.

In order to ascertain as far as possible the actual p_H of the solution used by Kendall and Nord we prepared a solution of reduced indigo by their procedure, using the exact quantities given in their paper. After reoxidising and filtering off the indigo the filtrate was found to be alkaline to alizarin (which behaves normally in such solutions); and its p_H must therefore have been at least 12, although it appeared to be neutral to phenol red. With phenolphthalein the maximum red colour was first produced, but this colour vanished almost instantaneously, owing to the reducing properties of the solution.

One of the main points of Kendall and Nord's paper was that in presence of the oxygen addition compound the reduction-potential was determined by the ratio of the concentrations of the —SH compound to the —SS— compound, *i.e.* there was a truly reversible system. Examination of their experimental evidence for this view fails however to establish their conclusion.

Taking a constant amount of cysteine in four electrode vessels, and oxidising varying proportions to cystine by adding different quantities of hydrogen peroxide in the presence of indigo carmine, Kendall and Nord found that the reduction-potential was highest where the ratio —SH : —SS— was highest, and varied directly with this ratio. Without further discussion they state that "it is therefore evident that the ratio of cystein to cystin establishes the equilibrium point in these solutions."

Since Dixon and Quastel found that the potential was determined solely by the concentration of the —SH compound, and that the —SS— group is without effect, it is obvious that varying the —SH concentration in the manner described by Kendall and Nord will result in variations of the reduction-potential in the sense observed by them. Any conclusion as to the importance of the *ratio* is fallacious unless the concentration of the —SH group is maintained constant and the concentration of the —SS— group varied, an experiment of which Kendall and Nord make no mention. Kendall and Nord themselves state that the —SS— form does not affect the electrode, thus showing clearly that the potential is determined solely by the —SH concentration and not by the ratio of this to the —SS—. The results obtained by Dixon and Quastel adequately explain the above observations.

Thus it appears that Kendall and Nord have brought forward no evidence of the reversibility of the —SH, —SS— system.

We do not propose to attempt any detailed discussion of the elaborate theories put forward by Kendall and Nord to account for the apparent properties of the hypothetical addition compound. We have confined ourselves in this paper to a consideration of the main points on which these theories are based. In any case it is sure that their results can have but limited application, since they refer only to the reduction of the particular dye indigo carmine, and do not occur with other dyes such as methylene blue. Still less could they have significance in tissue oxidation processes. The claim of Kendall and Nord that the active addition compound of glutathione with oxygen is the *sine qua non* for its physiological activity is not justified by the evidence.

SUMMARY.

There is no necessity for Kendall and Nord's assumption of an active addition compound of the —SH group with oxygen.

Their experiments do not show that the —SH, —SS— system is reversible.

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CXV. NOTE ON THE ERLENMEYER AMINO-ACID SYNTHESIS.

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THE series of reactions, constituting a general synthesis of α-amino-acids, devised by Erlenmeyer [1893] and since employed by many other workers, can be conducted, in most cases, with little trouble and excellent yields except at one stage. The condensation of an aromatic aldehyde with hippuric acid and the conversion of the resulting azlactone into the corresponding α-benzoylaminoacrylic acid proceed almost always in yields of 70 % or over. The ease with which the acrylic acid derivative may be reduced to the corresponding propionic acid varies considerably, however, in different cases. Moreover, even in those cases in which the ultimate yield obtainable is fairly high, the reaction is apt to be somewhat troublesome to carry out. In order to bring about this reduction, Erlenmeyer employed sodium amalgam, in which he has been followed by the majority of subsequent workers, although occasionally [cf. Barger and Ewins, 1917] sodium in alcohol has been used with success.

In the synthesis of thyroxine [Harington and Barger, 1927] where it was desired to utilise Erlenmeyer's method for the preparation of β-3 : 5-diiodo-4-[4′-(hydroxyphenoxy)phenyl]-α-aminopropionic acid, the use of any alkaline reducing agent was precluded by the presence of iodine atoms in the molecule. We attempted at first to surmount the difficulty by using hydrogen and palladium in neutral or acid solution, but our efforts in this direction were entirely without success. It then occurred to us that the desired end might be achieved by the use of hydriodic acid and red phosphorus, since we had previously obtained evidence that the iodine atoms, in the compounds with which we were working, were stable towards these reagents. This experiment was successful, and we were able, by boiling the α-benzoylamino-3 : 5-diiodo-4-(4′-methoxyphenoxy)cinnamic ester with hydriodic acid and red phosphorus, to obtain a 25 % yield of the desired amino-acid.

Later it became necessary, for further work, to prepare larger quantities of this amino-acid, and experiments were therefore undertaken with the object of improving the yield. Without going into the various unsuccessful variations that were tried, it may be said at once that we have succeeded in improving the above-mentioned yield of 25 % to one of 82 % by the simple modification of substituting for the constant boiling hydriodic acid originally employed a mixture of equal parts of the latter and of acetic anhydride. This mixture is an excellent solvent, and, by its use, the reaction could be made to proceed smoothly and to yield a clean product which could be worked up without difficulty. It may further be remarked that the whole reaction, *i.e.* the reduction and the simultaneous hydrolytic removal of the benzoyl group, was complete after boiling for 1½ hours with the hydriodic acid-acetic anhydride mixture, so that the saving of time effected, as compared with Erlenmeyer's original method, even supposing the latter to have been applicable in this case, was very considerable.

The surprising success of this method in the case under discussion, and its great rapidity, led us to investigate its applicability to the synthesis of some other amino-acids. In particular were we interested to try to prepare in this way 3 : 4-dihydroxyphenylalanine, the synthesis of which by Erlenmeyer's method has been carried out by Funk [1911], but with unsatisfactory yields. Using vanillin as our starting point we were indeed able to prepare this amino-acid in yields which were satisfactory, whilst not being so good as those which we obtained in other cases. We have further applied the method to the synthesis of phenylalanine, tyrosine, and desiodothyroxine, the results in all cases being superior to those obtainable by Erlenmeyer's original method.

It will be noticed in the experimental part that we have employed in some instances the benzoylaminoacrylic acid and in others the ester; we have observed slight but definite differences in the yields of the different amino-acids obtainable from the acid and the ester; it appears to be a matter of experiment to determine, in any given case, which is the more suitable.

EXPERIMENTAL.

β-[3 : 5-*Diiodo*-4-(4′-*hydroxyphenoxy*)*phenyl*]-*α*-*aminopropionic acid.*

α-Benzoylamino-3 : 5-*diiodo*-4-(4′-*methoxyphenoxy*)*cinnamic acid*. The azlactone prepared by the condensation of 3 : 5-diiodo-4-(4′-methoxyphenoxy)-benzaldehyde with hippuric acid [cf. Harington and Barger, 1927] was dropped into 100 parts of a boiling solution of 1 % sodium hydroxide in 70 % alcohol; the substance passed rapidly into solution and the reaction was complete after boiling for 5 minutes. The hot solution was acidified with hydrochloric acid, whereupon the acid began immediately to crystallise; after standing a few hours in the ice-chest, the acid was filtered off: the first crop so obtained amounted to 60 % of the theoretical yield; a further 30 % was obtained by heating the filtrate to boiling, diluting with an equal volume of hot water, and again cooling; the total yield was therefore almost quantitative. The acid was moderately soluble in hot alcohol, and sparingly soluble in water; on recrystallisation from 90 % alcohol it formed colourless needles, M.P. 239–241° (decomp.).

Analysis. 18·3 mg. gave 0·38 mg. N (micro-Kjeldahl).

Calculated for $C_{23}H_{17}O_5NI_2$ 2·2 % N; found 2·1 % N.

β-[3 : 5-*Diiodo*-4-(4′-*hydroxyphenoxy*)*phenyl*]-*α-aminopropionic acid.* 5 g. of the foregoing acid were boiled for 1¼ hours under a reflux condenser with a mixture of 25 cc. hydriodic acid (Sp. Gr. 1·7) and 25 cc. acetic anhydride together with 3 g. red phosphorus. The solution was filtered hot through asbestos into a Claisen flask, the phosphorus being washed with acetic acid. The filtrate was evaporated to dryness *in vacuo*; some water was added and the evaporation repeated. The residue was dissolved in about 40 cc. boiling water; the solution was cooled under the tap, the flask being well shaken, and the precipitate, consisting of the hydriodide of the amino-acid mixed with benzoic acid, was filtered off and washed thoroughly with ether; the aqueous portion of the filtrate was extracted twice with ether, and warmed to remove dissolved ether. The first crop of hydriodide was added to the boiling aqueous solution, a little hydrochloric acid being added, if necessary, to obtain complete solution, and the free amino-acid precipitated by the cautious addition of ammonia; 3·35 g. of the pure amino-acid, or 82 % of the theoretical amount, were obtained.

Phenylalanine.

10 g. of *α*-benzoylaminocinnamic acid, obtained by the method of Erlenmeyer [1893], were boiled under a reflux condenser with 100 cc. of the hydriodic acid-acetic anhydride mixture and 10 g. of red phosphorus for 1½ hours; the solution was filtered and evaporated to dryness *in vacuo*; water was added and the evaporation repeated; the residue was shaken up with water and ether; the aqueous layer was separated, and, after a second extraction with ether, was heated to boiling and carefully neutralised with ammonia; on cooling there separated, in colourless plates, analytically pure phenylalanine. The yield was 88 % of the theory.

Analysis. 8·8 mg. gave 0·74 mg. N (micro-Kjeldahl).

14·19 mg. gave 2·0 cc. moist N_2 at 19° and 766 mm. (Van Slyke).

	Total N	NH_2-N
Calculated	8·5 %	8·5 %
Found	8·4	8·1

Tyrosine.

In a precisely similar manner there was obtained from ethyl *α*-benzoylamino-*p*-methoxycinnamate a 60 % yield of tyrosine.

Analysis. 15·5 mg. gave 1·2 mg. N (micro-Kjeldahl).

14·11 mg. gave 1·83 cc. moist N_2 at 19° and 766 mm. (Van Slyke).

	Total N	NH_2-N
Calculated	7·7 %	7·7 %
Found	7·7	7·4

3 : 4-*Dihydroxyphenylalanine.*

Azlactone from vanillin and hippuric acid. An intimate mixture of vanillin (15·2 g.), hippuric acid (17·9 g.), and freshly fused sodium acetate (15 g.) was treated with 30 cc. acetic anhydride and heated on the water-bath for

15 minutes. The reaction product was ground up with water, filtered, and the precipitate well washed. The crude product was crystallised from glacial acetic acid, and formed yellow needles, M.P. 189°. The yield was 75 %.

Analysis. 15·2 mg. gave 0·646 mg. N (micro-Kjeldahl).

Calculated for $C_{19}H_{15}O_5N$ 4·15 % N; found 4·25 % N.

Ethyl α-benzoylamino-3-methoxy-4-hydroxycinnamate. 10 g. of the above azlactone were dissolved in 100 cc. alcohol to which were added 10 cc. concentrated sulphuric acid, and the solution was boiled under a reflux condenser for 15–20 minutes; the greater part of the alcohol was then distilled off under reduced pressure, and the residue was poured into a dish and allowed to stand. After some days crystallisation began and, at the end of a week, was complete; the ester was filtered off and recrystallised from dilute alcohol. It formed colourless prisms, M.P. 128–129°. The yield was 65 % of the theoretical amount.

Analysis. 0·0862 g. gave 0·2109 g. CO_2; 0·0450 g. H_2O.

15·7 mg. gave 0·63 mg. N (micro-Kjeldahl).

	C	H	N
Calculated for $C_{19}H_{19}O_5N$	66·8 %	5·6 %	4·1 %
Found	66·7	5·8	4·0

It will be noted that the analysis indicates that the acetyl group, which was present in the azlactone, was split off in the process of preparation of the ester.

3 : 4-*Dihydroxyphenylalanine.* 5 g. of the ester were boiled for 1½ hours with 25 cc. hydriodic acid, 25 cc. acetic anhydride and 5 g. red phosphorus, in an atmosphere of hydrogen; the solution was filtered and evaporated to dryness under diminished pressure, hydrogen being led into the capillary tube of the Claisen flask; the aqueous solution of the residue, after removal of the benzoic acid by ether extraction, was neutralised with ammonia and evaporated to dryness under the same precautions to avoid access of air; the product was dissolved in a little water and the solution treated with a large excess of alcohol which precipitated the greater part of the pigment; the latter was rapidly filtered off and the filtrate once more taken to dryness. The residue was dissolved in hot water containing a little sulphur dioxide, the solution was boiled with charcoal and filtered, and the filtrate allowed to crystallise in a vacuum desiccator. In this way the amino-acid was obtained almost colourless and in well-formed crystals. The yield was 50 % of the theoretical. The substance melted at 269° (decomp.) and agreed in its properties with the product described by Funk [1911].

Analysis. 14·3 mg. gave 0·98 mg. N (micro-Kjeldahl).

21·3 mg. gave 2·55 cc. moist N_2 at 17° and 760 mm. (Van Slyke).

	Total N	NH_2-N
Calculated	7·1 %	7·1 %
Found	6·9	6·9

Desiodothyroxine.

The azlactone was prepared in the usual manner from 4-(4′-methoxyphenoxy)benzaldehyde [cf. Harington, 1926] and hippuric acid; there was obtained 70 % of the theoretical amount of a substance which, after crystallisation from glacial acetic acid, formed yellow needles, M.P. 141°.

Analysis. 16·0 mg. gave 0·575 mg. N (micro-Kjeldahl).

Calculated for $C_{23}H_{17}O_4N$ 3·8 % N; found 3·6 % N.

The above azlactone was converted into the acid by boiling for 5 minutes with 100 parts of 1 % sodium hydroxide in 45 % alcohol; the acid, on recrystallisation from dilute alcohol, formed colourless branched needles, M.P. 192·5°. The yield was 95 % of the theory.

Analysis. 15·8 mg. gave 0·554 mg. N (micro-Kjeldahl).

Calculated for $C_{23}H_{19}O_5N$ 3·6 % N; found 3·5 % N.

The preceding compound was converted into the amino-acid in a precisely similar manner to that described for tyrosine and phenylalanine. The yield of amino-acid was 61 % of the theoretical. The product melted at 253–254° (decomp.), and gave a hydrochloride with M.P. 239–240°; it was therefore in all respects identical with the substance previously prepared by Harington [1926].

Analysis. 19·9 mg. gave 1·02 mg. N (micro-Kjeldahl).

26·8 mg. gave 2·22 cc. moist N_2 at 17° and 760 mm. (Van Slyke).

	Total N	NH_2-N
Calculated for $C_{15}H_{15}O_4N$	5·1 %	5·1 %
Found	5·1	4·9

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CXVI. THE ENZYMIC DEAMIDATION OF ASPARAGINE IN THE HIGHER PLANTS.

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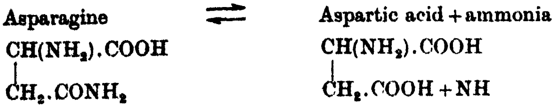
THE occurrence of asparagine in the higher plant and its rôle in protein metabolism have formed the subject of numerous investigations.

It has been found that the amount normally present in the green plant is very small and it is only under artificial conditions, associated with a carbohydrate deficiency, that an accumulation of this substance is ever obtained. E. Schulze [1906], in a series of papers, showed that in etiolated seedlings most of the protein reserve is converted into asparagine. In the mature plant, in which the leaf is the seat of metabolic activity, it has been found since Schulze's work that, in general, starvation results in a conversion of protein to asparagine.

Chibnall [1924], in the course of investigations on the nitrogenous metabolism of the higher plants, concludes that there is a constant production of asparagine from protein in the normal mature leaf, and that in plant metabolism asparagine has the rôle of a translocatory substance and is the chief medium whereby nitrogen, in a form suitable for subsequent resynthesis, can be conveyed from one part of the plant to another. According to Prianischnikov [1916] asparagine is formed as a temporary nitrogen reserve whenever there occurs, through some derangement of the normal course of protein metabolism, an excess of ammonia which is toxic to the plant.

Important as is the part played by asparagine in protein metabolism, the reactions whereby protein and asparagine are interconverted are still far from being clearly understood. On the one side the degradation of protein through proteoses and polypeptides to amino-acids is well known, and a similar reverse process is probable; but there is little or no evidence regarding the steps intervening between these amino-acids and asparagine. It has been assumed that the intermediate forerunner of asparagine in the plant is aspartic acid, and that an enzyme, for which the name asparaginase has been used, controls the reaction



$$\begin{array}{lcl} \textbf{Asparagine} & \rightleftharpoons & \textbf{Aspartic acid + ammonia} \\ CH(NH_2).COOH & & CH(NH_2).COOH \\ | & & | \\ CH_2.CONH_2 & & CH_2.COOH + NH \end{array}$$

The presence of aspartic acid in the plant had never been demonstrated until Kiesel [1924] claimed to have isolated it in the course of a separation of the nitrogen compounds of ripening rye ears. As no asparagine was found even in the later stages of ripening, Kiesel considered that aspartic acid cannot be the forerunner of asparagine. It is probable, however, that the reverse reaction is here occurring—that there is a breakdown of asparagine to aspartic acid; but instead of the deamidation only taking place at a stage still earlier than those investigated, as Kiesel suggests, it is more likely that there is a continuous diffusion of asparagine into the grain with almost simultaneous deamidation. Schulze and Winterstein [1910] came to the conclusion that asparagine migrates into the ripening seed where it is rapidly utilised for the production of protein.

In the earlier experiments on the autolytic splitting of ammonia from plant material, the whole process was referred to as one of "desamidation," and the inability to distinguish between the processes of deamination and deamidation, *i.e.* the removal of ammonia from the amino- and amide-groupings respectively, has given rise to considerable confusion and controversy. The enzymes responsible for the reactions leading to the liberation of ammonia were indiscriminately termed amidases or de-amidases, and these expressions are still often employed.

Many attempts have been made to effect a definite enzymic deamidation of asparagine by means of plant material. The only instance, however, in which this is claimed to have been achieved is that recorded by Kiesel [1909]. By the autolysis of etiolated seedlings or of the expressed juice, the whole of the asparagine originally present was broken down. The time period of four months, however, was somewhat excessive, for asparagine solutions, especially in the presence of plant material, are extremely susceptible to bacterial infection. It was not stated whether any sterility tests were carried out at the end of autolysis. The products formed by the breakdown of the asparagine were not identified and the attempt to obtain a preparation active on asparagine by precipitation of the expressed juice with alcohol was unsuccessful.

Schulze and Winterstein [1910] tried to detect in unripe pea and bean seeds an enzyme which would liberate ammonia from asparagine, but without success; in both cases practically the same amount of asparagine was recovered in the active and control experiments. Kato [1911] found that the press-juice of bamboo shoots contained an enzyme which would liberate ammonia from asparagine. The amount formed, however, was almost inappreciable, being of the order of 0·3 mg. from 0·2 g. of asparagine after 50 hours' incubation.

The presence of "amidases" in moulds was first demonstrated by Shibata [1904] who found in the mycelium of *Aspergillus niger* an enzyme which acted on certain amides including asparagine. The ammonia set free in the case of asparagine was only about 0·5 %. Pringsheim [1908], using an acetone-dried preparation of yeast, was unable to obtain any "desamidation" of amino-acids or asparagine and considered that the ability of moulds to liberate ammonia from nitrogenous substances was essentially associated with a living condition of the mould.

The "de-amidases" of yeast were briefly dealt with by Dernby [1917] in connection with a study of the yeast proteolytic enzymes. Yeast press-juice was found to be inactive on amino-acids but asparagine was readily broken down. As the ammonia formed was always less than 50 % of the total nitrogen, it was concluded that it had been set free by the yeast ereptase from the —$CO.NH_2$ group which was regarded as a peptide linkage. This point will be discussed later in connection with some results obtained in the present research.

Effront [1917], in the course of a discussion on amidases, describes the preparation of a "butyro-amidase" which acted on acetamide and asparagine with liberation of ammonia. When a solution of asparagine only was used, the action was limited to the amide group; if, however, oxidisable substances were also present—*e.g.* products of proteolysis—then the amino-group of the asparagine was also attacked. This is one of the very few doubtful instances in which the amino-group has been considered to have been displaced by enzyme activity apart from the living cell. In the rather vague account of his experiments on butyro-amidase, Effront makes no reference, unfortunately, to the original publication of the results which are quoted.

In the sphere of animal physiology, the earliest investigations on the existence of amidases in the tissues of the animal organism were attended by the same lack of differentiation between deamination and deamidation; resulting from this, divergences of opinion and conflicting results are again observed. S. Lang [1904] found that minced organs were able to effect the removal of ammonia not only from asparagine but also from certain amino-acids. Abderhalden and Schittenhelm [1906], using the press-juice from liver, obtained no action on amino-acids. Fürth and Friedmann [1910], experimenting with various organs of the horse and pig, found that, in general, they were all capable of bringing about *in vitro* the hydrolysis of the amide group of asparagine. Further, the amounts of ammonia formed were usually no greater than could be accounted for by the amide nitrogen. It was suggested that the ability to break down asparagine was related to the general autolytic processes taking part in the physiological degradation of proteins.

More recently, Clementi [1922] carried out a systematic study of the enzymic deamidation of asparagine by the animal organism. Contrary to the findings of Fürth and Friedmann, Clementi asserts that the enzyme, for which he uses the name "asparaginase," is present only in certain animals and in certain tissues. It is interesting to note that whereas Fürth and Friedmann obtained the maximum activity with intestinal mucous membrane of the horse, Clementi, using the same material, found only a slight deamidation. The intestine of the pig was found to be quite inactive, but this result was not confirmed in the present research. With regard to the physiological significance of the appearance of asparaginase in the animal organism, Clementi elaborated the theory that this enzyme is related, not to the processes of metabolic degradation of the protein molecule, but rather to the nature of the alimentation of the species, and that the phenomenon is one of biochemical adaptation of the organism to the presence or absence of asparagine in the food. The experimental data obtained by Clementi appear to conform very well to this attractive hypothesis.

From the above introductory review it is apparent that the only clear-cut evidence for the occurrence of the enzymic deamidation of asparagine apart from the living cell is that obtained by experiments with animal material. Even these investigations, however, have had as their object merely the proof of the existence of an enzyme capable of liberating ammonia from asparagine. No attempt has been made to furnish experimental proof that the product of the deamidation of asparagine is aspartic acid and, except in the researches of Clementi, no active preparation of the enzyme by alcohol or acetone precipitation has been obtained. Further, the factors which condition the activity of the enzyme have received little attention. Clementi found that a slightly alkaline medium was favourable but did not determine the optimum p_H for the enzyme or the range over which it was active.

In the higher plant even the existence of this enzyme has not yet been satisfactorily demonstrated, although the deamidation of asparagine is a phenomenon of the greatest importance in the metabolic processes of the plant. The primary object of this research, therefore, was to prove conclusively the presence of this enzyme in the higher plant; a further consideration was the need of obtaining some information as to the nature of the enzyme.

EXPERIMENTAL.

General methods.

In the following experiments, the young rootlets (culms) of germinated barley were used as the material from which to obtain the enzyme and investigate its nature. The barley was obtained from the malthouse (Messrs Watney, Combe and Reid, Mortlake) in the fresh or "green" condition, *i.e.* before the kilning process. It had been allowed to germinate 8–9 days and the rootlets were 1–1·5 cm. long. For the experiments on asparagine, a $N/10$ solution containing 15 g. per litre was used throughout; the solution contained 0·5 % cresylic acid (tricresol) as antiseptic except where otherwise stated. Cresylic acid was used in preference to toluene partly because the trouble of separating the toluene before estimation was avoided and, more especially, because it had been found by other investigators to be more efficient. In general, the experiments were carried out at 37° and the ammonia formed was estimated by distillation with magnesia and absorption of the ammonia in $N/10$ HCl.

The amount of asparagine broken down in the course of the distillation is very small; for 10 cc. $N/10$ asparagine the blank titration is approximately only 0·3 cc. This was always accounted for in the parallel control experiments which were carried out under the same conditions except that the enzyme had been destroyed by previously heating the material.

Activity of barley culms on asparagine.

For a preliminary test of activity, 4 g. of fresh roots were picked off from 100 g. of the barley and ground to a thin cream with water to 25 cc. The activity of the fresh roots is shown in Table I, 5 cc. of the preparation being equivalent to 0·8 g. of roots. As the separation of the fresh culms by hand picking was an extremely tedious and lengthy process, the remainder of the germinated barley was spread out in an incubator at 37° and allowed to dry for 3 days. The dried culms were then fairly easily separated by vigorously shaking the barley and afterwards sifting through a coarse mesh. From 9·5 kg. of the dried barley, 200 g. of culms were obtained and ground to a fine powder. By comparison with the yield of fresh culms, it is seen that the culms had lost at least half their weight by drying. If the enzyme had been unaffected by the drying process, therefore, the dried culms should have had about double the activity of the same weight of fresh culms. Actually the activity of the dried culm powder was certainly no greater than that of the same weight of fresh material. 10 g. of the root powder were ground to a thick paste with water to 80 cc. and 5 cc. (= 0·6 g. of roots) used for the activity tests given in Table I.

Table I. *Activity of fresh and dried barley roots on asparagine.*

(Maximum ammonia formation = 10 cc. $N/10$.)

	Time (hours)	cc. $N/10$ NH_3
Fresh roots:		
5 cc. preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	88	8·75
5 cc. preparation + 10 cc. 0·5 % tricresol + 5 cc. buffer p_H 7·5	88	0·7
5 cc. (boiled) preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	88	0·6
Dried roots:		
5 cc. preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	88	7·5
5 cc. preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	161	8·55
5 cc. preparation + 10 cc. 0·5 % tricresol + 5 cc. buffer p_H 7·5	161	1·45
5 cc. (boiled) preparation + 10 cc. $N/10$ asparagine + 5 cc. buffer p_H 7·5	161	1·25

Enzyme preparation by alcohol precipitation. 200 g. of dried barley culms were ground with 1½–2 litres of water for about 2 hours, sand being added to facilitate the grinding. The mass was wrapped in a thick filter-cloth and pressed out in a Buchner press. The residue was returned to the mill, ground with a further 1½ litres of water for 1 hour and again pressed out. The combined extracts measuring 2½ litres were centrifuged, and the clear liquid was precipitated with 4½ litres of ice-cold 90 % alcohol. The precipitate was centrifuged off and washed with absolute alcohol and twice with ether, being centrifuged after each washing. The precipitate was air-dried as far as possible by breaking up in a small mortar, and the last traces of ether removed in a vacuum desiccator. Those steps in which the enzyme was in the presence of alcohol were carried out as rapidly as possible in order to minimise inactivation. The pale creamy-brown powder thus obtained weighed 8·85 g. or 4·4 % of the original dried root material; it was only partially soluble and when ground with water gave a thin suspension which slowly deposited. This alcohol precipitate is referred to below as the enzyme preparation.

To determine the activity of the preparation, 0·5 g. were ground with water to 25 cc. and 5 cc. of this suspension (= 0·1 g. of preparation) used for each of the experiments represented in Table II. Since 0·5 g. of the dried roots used for this preparation produced a break-down of 5·8 cc. $N/10$ (corrected) in 144 hours under the same conditions, it can be roughly calculated that the activity of the enzyme preparation was about 4–5 times that of the whole roots.

Table II. *Activity of the enzyme preparation.*

(Maximum ammonia formation = 10 cc. $N/10$.)

No.		Time (hours)	cc. $N/10$ NH_3
1.	5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	50	3·0
2.	5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	118	5·3
3.	5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	170	6·7
4.	5 cc. suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	218	7·8
5.	5 cc. (boiled) suspension. 10 cc. asparagine. 5 cc. buffer p_H 7·5	218	0·8

The activity of the enzyme preparation under sterile conditions was confirmed, both in the presence of toluene and of cresylic acid. The extent of hydrolysis was practically the same as that obtained under the ordinary experimental conditions. The sterility tests were kindly carried out by Dr Wright and Mrs Channon of the Bacteriological Department, University College Hospital Medical School.

Isolation of aspartic acid as the end-product of the deamidation of asparagine. 2 g. of the enzyme preparation ground with water were added to 150 cc. $M/5$ asparagine solution (containing 0·5 % tricresol) together with 1·5 cc. of 2 N Na_2CO_3. The volume was made up to 200 cc. in a stoppered measuring cylinder and incubated at 37°. 5 cc. portions were drawn off and estimated at intervals until the maximum theoretical amount of ammonia was nearly obtained.

The results of these determinations are given in Table III; the action is slower than usual and this may be due to the higher concentration of tricresol in the mixture. The values for the velocity constant (monomolecular), calculated after allowing for an arbitrary blank of 0·6 cc., are also given. The slight fall in the values has been noted in the case of other enzymes, *e.g.* trypsin and lactase, and is due in part to the effect of the products of the reaction.

Table III.

Time (hours)	cc. $N/10$ NH_3 (max. = 7·5 cc.)	k
46	1·6	229×10^{-5}
90	2·3	207
164	3·3	192
260	4·5	189
380	5·7	188
520	6·65	179
640	7·35	176

At the end of the 27 days, the enzyme material was precipitated from the remainder of the mixture by addition of alcohol. The solution was evaporated to about 50 cc. and the aspartic acid precipitated with the requisite amount of HCl. After standing in the ice-chest overnight, the aspartic acid was filtered off, washed with 50 % alcohol, absolute alcohol and ether successively, and finally dried in a desiccator. 1·96 g. were thus obtained. By concentrating the mother-liquor and adding an equal volume of alcohol, a further 0·46 g. was obtained. The total yield was therefore 2·42 g. or 76 % of the theoretical 3·2 g. The loss is due in part to an incomplete separation of the aspartic acid and possibly, also, to anhydride formation. The crude aspartic acid on recrystallisation gave 2·16 g.

Analysis.

1. *Amino-nitrogen* (Van Slyke). Found 10·38 %, calculated 10·53 %.
2. *Total nitrogen* (Kjeldahl). Found 10·57 %, calculated 10·53 %.
3. *Formaldehyde titration* (method of L. G. Harris). The solution of aspartic acid used contained 0·4018 g. per 100 cc.

	cc. $N/10$ NaOH titrating to phenolphthalein
1. COOH *estimated*	
(*a*) 5 cc. of aspartic acid solution + 50 cc. alcohol + 10 cc. neutralised formaldehyde	2·92 (corr.) Theory 3·02

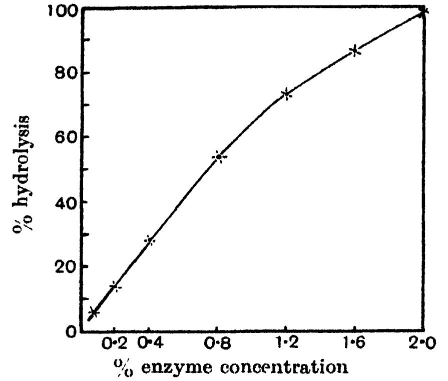
	cc. $N/10$ HCl titrating to methyl red
2. NH_2 *estimated*	
(*b*) Back titration of solution (*a*)	1·4 (corr.) Theory 1·51

Relation between quantity of enzyme and extent of hydrolysis. Fig. 1 shows the curve obtained by plotting the corrected values of the percentage hydrolysis against enzyme concentration. The extent of hydrolysis is fairly directly proportional to the quantity of enzyme preparation, but the curve falls off slightly at the higher concentrations.

Effect of temperature on activity. For this experiment a new enzyme preparation was used, 0·1 g. being taken for each estimation; both active and control experiments were carried out in duplicate at each temperature.

The curve obtained by plotting the corrected mean values of the percentage hydrolysis is shown in Fig. 2. The preparation used was unfortunately of a low activity but the curve shows that the optimum temperature for the enzyme is about 38°. The time period for this curve was 120 hours.

Effect of p_H on activity. As this series was started at the same time as the previous one, the same preparation was used, but the time of incubation was increased to 12 days. 0·1 g. of enzyme ground with water to a thin suspension was added to 10 cc. of the asparagine solution and 5 cc. of the buffer indicated in each case. The actual p_H of each mixture was determined electrometrically after equilibrium had been reached in the course of some hours. The preparation had a very high buffering power, this being the more in evidence the further the p_H from neutrality. The optimum p_H was found to be approximately 7; the phosphate buffer was more favourable for the enzyme than the phthalate or borate buffers at the same p_H. Fig. 2 shows the curve obtained by plotting the actual p_H values against the corrected mean figures for percentage hydrolysis.



(Time = 63 hours)

Fig. 1.

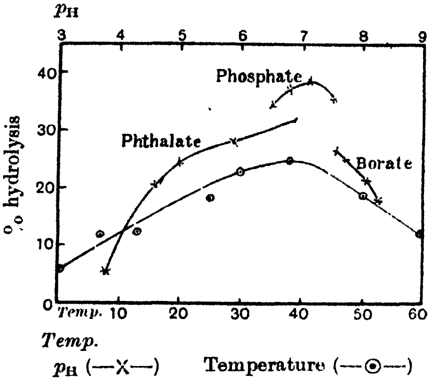


Fig. 2.

Specific action of the enzyme preparation on l-*asparagine.* The asparagine used in the previous experiments was the naturally occurring laevo-form with which the enzyme normally has to deal. It was interesting to find out whether the enzyme was specific for this form or was also capable of effecting the hydrolysis of the dextro-modification. It has been shown by Bergell and Wülfing [1910] that the laevo-component of *dl*-leucinamide is hydrolysed by pancreatin very much more readily than the dextro-form.

A preparation of asparagine containing an excess of the dextro-component was prepared according to the method of Pringsheim [1910]. The rotation in 10 % HCl was $[\alpha]^{18°}_{\text{Hg green}} = -11{\cdot}31°$ as compared with $+38{\cdot}8°$ for the natural laevo-form. Table IV shows the hydrolysis of a solution containing 0·6202 g. per 50 cc. by a water extract of the whole barley roots. Only about 50 % of the maximum ammonia formation was obtained, and it would appear

Table IV. *Activity of enzyme preparation on a mixture of* d- *and* dl-*asparagine.*

(Maximum ammonia formation = 4·2 cc. $N/10$.)

Time (hours)	cc. $N/10$ NH_3 Active	Control	Corrected
44	4·4	2·6	1·8
68	4·6	2·6	2·0
92	4·7	2·6	2·1
212	4·8	2·6	2·2

that the enzyme has little or no activity on the dextro-form of asparagine. Ravenna and Bosinelli [1919] showed that when moulds are grown on a mixture of *d*- and *l*-asparagine, the laevo-form is readily attacked but the dextro-form is unaffected.

The specificity of the enzyme preparation and the nature of deamidation. The simple amides, acetamide and propionamide, were entirely resistant to the action of the enzyme, but *d*-glutamine, which is closely associated with asparagine both in structural form and in its natural occurrence, was hydrolysed fairly rapidly. In these experiments the ammonia estimations were carried out by aeration with potassium carbonate in the cold. It is interesting to note that urea also was broken down but at a very much slower rate than with soya-bean urease.

With the object of obtaining some information on the nature of the enzyme and of the process of deamidation, the experiments described below were carried out. In order to ascertain whether deamidation is related to the processes underlying protein degradation, the action of the proteolytic enzymes pepsin, trypsin and, more particularly, erepsin was investigated.

Pepsin and trypsin were both found to be inactive on asparagine; the inactivity of trypsin had been previously demonstrated by Schwarzschild [1904].

A preparation of pig erepsin, however, proved to be readily active on asparagine, although Clementi's results with this material were negative. Bergell and Wülfing [1912] in their experiments on the splitting of leucineamide by pancreatin concluded that there was present a specific enzyme responsible for the amide cleavage. Levene, Simms and Pfaltz [1926], using intestinal erepsin, were able to obtain a slight hydrolysis of glycineamide. In a recent study of the peptidases, Waldschmidt-Leitz and his collaborators [1927] found that pig erepsin had a slight activity on glycineamide but hydrolysed leucineamide fairly readily.

The results obtained by acting on asparagine with an aqueous-glycerol extract of the mucous membrane of pig intestine are given in Table V. The p_H values in each case were measured electrometrically. It is seen that below p_H 6 the enzyme has only a very slight activity and this is the minimum p_H for the action of erepsin on dipeptides. The control figures were obtained by using the same amount of boiled extract.

Table V. *Action of pig erepsin on asparagine.*

(Maximum ammonia formation = 10 cc. $N/10$.)

		cc. $N/10$ NH_3		
p_H	Time (hours)	Active	Control	Corrected
3·0	68	2·15	2·2	0
4·0	68	2·35	2·2	0·15
5·1	68	4·0	2·2	1·8
6·0	68	7·4	2·2	5·2
7·1	50	7·4	2·25	5·15
8·0	50	8·7	2·25	6·45

From a consideration of these results, the question arises whether the enzyme in the barley preparation responsible for the splitting of the amide group of asparagine is merely a plant erepsin or—to use the preferable nomenclature—a peptidase. The action of the barley enzyme on a dipeptide (glycylglycine) was therefore investigated.

Activity of the barley enzyme towards glycylglycine.

For each estimation 10 cc. of barley root extract (containing 0·2 % tricresol) were added to 2 cc. of $M/10$ glycylglycine together with 0·25 cc. of $N/10$ NaOH to raise the p_H to 7·6. At the end of the indicated time at 37°, 40 cc. of alcohol were added and the mixture filtered. The filtrate was titrated with $N/10$ NaOH forward to phenolphthalein and with $N/10$ HCl back to methyl red. Table VI shows the increase in the titration values over the initial values showing that the hydrolysis has proceeded up to 70 % of the theoretical maximum.

Table VI. *Action of barley enzyme on glycylglycine.*

(Maximum value for both titrations = 2 cc. $N/10$.)

Time (hours)	Titrations, cc. $N/10$	
	Forward	Back
0	0·0	0·0
20	0·6	0·55
24	0·75	0·65
48	0·95	1·85
72	1·30	1·20
96	1·50	1·30

DISCUSSION.

The results of the foregoing experiments prove beyond question the existence in the higher plant of an enzyme capable of effecting the deamidation of asparagine. The fact that its action had not previously been satisfactorily demonstrated is probably due to the comparatively low activity of the enzyme or to the difficulty of obtaining it in a sufficiently concentrated form.

With regard to its identity, the experiments on the hydrolysis of glycylglycine and on the behaviour of the proteolytic enzymes towards asparagine would seem to indicate that we are here dealing with an enzyme in the nature of a plant erepsin. This is in accordance with the suggestion made by Dernby from his results on the hydrolysis of asparagine by autolysed yeast. As was pointed out in the introduction, Dernby considered that the amide group of asparagine was to be regarded as similar to a peptide linkage and that it was the yeast ereptase which was responsible for its cleavage.

The enzyme which breaks down asparagine has hitherto been included in the group of amidases under the name of asparaginase. The nomenclature of this group is discussed by Oppenheimer [1926] who separates the amidases into two sections. Those which are responsible for the liberation of ammonia from amino-acids—and which have a very doubtful existence apart from the living cell—he terms *amino-acidases.*

The name *amidases* he retains for the enzymes which, in general, are capable of hydrolysing the —CO.NH$_2$ grouping only. There is, however, still no sharp line of demarcation between this group and the peptidases to which the cleavage of the peptide linkage —CO.NH— is due.

Levene and Waldschmidt-Leitz have both probably assumed the peptide nature of the amide group in studying the action of peptidases on such amides as glycineamide as well as on true dipeptides. By adopting the view that the amide group of asparagine is essentially a peptide linkage and that both asparagine and glycylglycine are hydrolysed by a barley peptidase, our conception of the protein metabolism of the plant is considerably simplified. Asparagine need then no longer be regarded as a substance apart and distinct from the usual products of protein metabolism but simply as a dipeptide.

If the deamidation of asparagine is indeed due to a peptidase, as is here considered to be the case, then the conclusions of Clementi on the specific distribution of "asparaginase" in the animal kingdom would have very little significance. Since erepsin is an essential factor in the protein metabolism of all animals, the ability to break down asparagine should be universal.

SUMMARY.

A review of the literature shows that in spite of the great importance of asparagine in plant metabolism the enzyme responsible for its deamidation had not been satisfactorily demonstrated.

This enzyme has been shown to be present in the rootlets of germinating barley, and an active preparation has been obtained by alcoholic precipitation of the aqueous extract. It appears to be specific for the naturally occurring *l*-asparagine. The optimum p_H is about 7 and the optimum temperature about 37°.

A preparation of pig intestinal erepsin was found to be active on asparagine. As the barley preparation is able to effect the hydrolysis of glycylglycine a peptidase (barley erepsin) must be present.

It is concluded that the asparagine is also hydrolysed by this enzyme, and that it is unnecessary to assume the presence of any specific amidase (asparaginase).

We wish to thank the Government Grant Committee of the Royal Society for a grant in aid of expenses.

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CXVII. NOTE ON THE UNSAPONIFIABLE MATTER OF YEAST-FAT.

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(*Received July 1st, 1927.*)

During an investigation on the phospholipins of yeast, the results of which were recently described by us [1927], we determined the iodine value of the total unsaponifiable matter of yeast fat. The particular specimen examined gave an iodine value of 95·5: about 50 % of this material consisted of a sterol precipitable by digitonin and giving iodine values lying between 188 and 200. We therefore made the deduction that the unsaponifiable matter consisted partly of sterol and partly of a saturated substance.

Further investigation of the unsaponifiable matter shows that this deduction is not generally true for most of the specimens of unsaponifiable material which have been subsequently examined. One reason for this is that the iodine values of the unsaponifiable matter vary widely according to the particular sample of yeast employed and according to the method used for the isolation of the unsaponifiable matter. After removal of the sterol precipitable by digitonin, an oil may be obtained giving a very high iodine value.

It was shown by Smedley-MacLean and Thomas [1920] that the iodine values of the sterols determined by Wijs' reagent gave variable values often twice as great as those determined by means of Hubl's reagent. Even with the latter reagent abnormally high values may be obtained, and our recent results have confirmed the opinion that, in this case too, variable results may be obtained unless the conditions are very carefully controlled.

That part of the unsaponifiable matter of yeast-fat which is not precipitated by digitonin appears to vary considerably in its composition, and its degree of unsaturation is very largely influenced by the particular method of saponification used and by the subsequent treatment the material undergoes. Other highly unsaturated and probably labile substances are present in varying proportion. An investigation is at present in progress which it is hoped will throw some light on the nature of the complex mixture of substances included under the designation of the unsaponifiable matter of yeast-fat.

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CXVIII. VITAMIN A FORMATION IN THE ETIOLATED WHEAT SHOOT.

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(*Received July 1st, 1927.*)

As the result of extensive feeding experiments with green plant tissues, etiolated tissues, and seeds, Coward and Drummond [1921] established the superiority of green tissues as a source of vitamin A (not then differentiated from vitamin D). Very small amounts of vitamin were found in seeds and in etiolated tissues, which led to the inference that vitamin A formation in the growing plant was to be associated with the presence of chlorophyll. The vitamin was to be considered a product of photosynthesis.

A year later Wilson [1922] contradicted this view. Experiments were described in which the dried powdered stems of wheat grown to a height of 3 in. in the dark, and fed to rats at rates of 5 and 8 % of the basal ration in preventive and curative experiments respectively, were found to be as effective as the same amounts of dried powdered green stems in permitting growth on a diet deficient in vitamin A. Wilson concluded that light was unnecessary for the formation of the vitamin, and that if green shoots contained more vitamin A than similar etiolated shoots this was in all probability due to the acceleration of the physiological processes of the plant during photosynthesis.

To these conclusions Coward [1923] advanced the objection that no control experiments had been carried out to test the vitamin content of the seeds from which Wilson's etiolated shoots had been derived, a criticism which gained weight through the presence in his basal diet of 20 % of lard, which might have contained traces of vitamin just insufficient to support growth, but sufficient when supplemented by traces of vitamin from the seeds. Coward still maintained that light was necessary for the formation of the vitamin, but as the result of further experiments admitted that the presence of chlorophyll was not essential. Vitamin A formation could proceed in the absence of carbon dioxide or oxygen, but was prevented by the presence of chloroform.

By some authorities [Accessory Food Factors Committee, 1924; McCollum and Simmonds, 1925] the results of Coward and of Wilson have been considered contradictory, but while this is true of the conclusions reached the actual experiments seem to have been quite incomparable. Wilson's 5 and 8 % allowances of powdered stems may be calculated to have been derived from wheat equivalent to 18 and 29 % of the diet, and on a food intake of 10 g. 125 and 200 stems must have been fed daily to each rat. In reply Coward quoted experiments in which the daily dose was limited to three etiolated shoots, or to the unsaponifiable matter from fifteen. Even at such a low level slight growth was observed in some cases.

Further experiments by Coward [1925] led to no change in her position, which was approved by Rosenheim and Drummond [1925]. Hess and Weinstock [1924], however, had shown that fresh etiolated wheat shoots, even when fed to rats at the rate of 10 g. daily, were ineffective in curing rickets, and it was obviously desirable to repeat the experiments with vitamin D, so intimately connected with sunlight, supplied by the irradiation of the diet, or some other means. By using a technique so modified and by increasing the daily allowance of shoots from three to five, Coward [1927] has recently detected the presence of appreciable quantities of vitamin A in etiolated wheat shoots, and has accordingly abandoned the view that light is necessary for the formation of the vitamin.

The experiments to be described, which were nearing completion when Coward's results were published, afford independent confirmation on this important point.

EXPERIMENTAL.

The wheat seeds used weighed on an average 20 to the g., and seem therefore to have been of a larger variety than those used by Wilson, which weighed 68 to the g. They were sprouted in moist sand contained in zinc trays, which were arranged in a wooden box. The box was painted black externally and the open side, by which the trays were inserted, was covered by a flap of four thicknesses of black calico, held in position by elastic bands. In the routine finally adopted all sowing, collection, and feeding was carried out after nightfall. Each tray accommodated seeds to provide shoots for 2 days' feeding. Sowings were accordingly performed on alternate days, the sand being strongly heated before each sowing to destroy bacteria. Sprouting was continued for 10 days, the shoots obtained varying somewhat in size in the different batches, but as a rule having stems about 4 in. long. In colour they were pale yellow.

Young rats, of weights from 42 to 60 g., were "run out" on the customary basal diet of this laboratory, 0·25 cc. of marmite extract and 0·2 cc. of lemon juice per rat per day supplying vitamins B and C respectively. Vitamin D was supplied by the administration of daily doses of 5 mg. of irradiated cholesterol from the points indicated on the accompanying growth curves. Out of a dozen rats the four weakest were used as positive controls (not shown), the remaining eight were given either etiolated shoots or seeds at the points indicated on the curves (Fig. 1), one bar corresponding to ten shoots or seeds, two bars to twenty, and three to thirty.

The four rats receiving seeds declined rapidly in weight, became rough-coated and emaciated, developed respiratory troubles and either died or were killed in dying condition. The rats receiving etiolated shoots, at first fed in a dark room in advance of the basal ration, remained comparatively smooth-coated. Three rats (nos. 2, 3 and 4) fluctuated in weight, though gaining on the whole. The fourth (no. 1) showed a serious decline and developed respiratory troubles, possibly through delay in feeding the shoots. Unfortunately some of the animals did not regularly ingest all the shoots offered, usually nibbling the grain and discarding the stem and roots: it therefore became necessary to modify the routine. Thirty entire shoots were now allowed to each animal, the stems, roots, and grains being finely chopped and mixed intimately with the basal ration, which was fed after nightfall and consumed before morning. In addition, the rats were irradiated before a mercury arc lamp for 10 minutes daily.

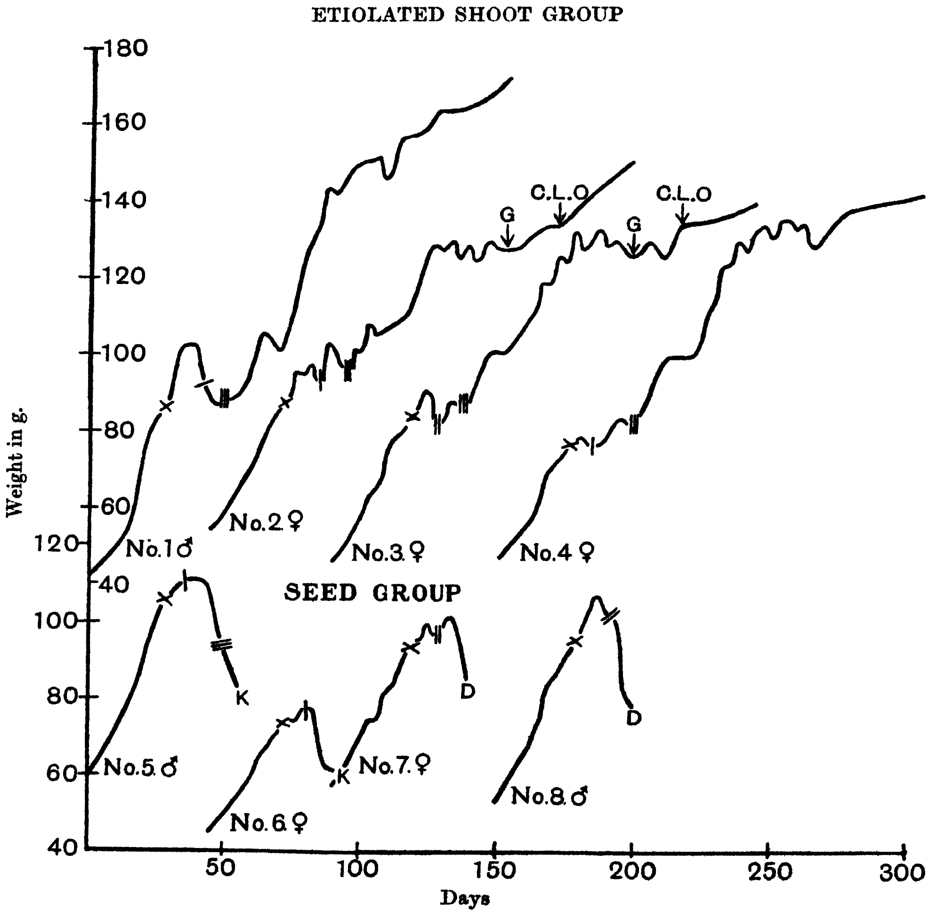

Fig. 1. Formation of vitamin A in the etiolated wheat shoot.

× = Feeding of irradiated cholesterol commenced.

|, ||, ||| = 10, 20 or 30 seeds or etiolated shoots fed *per diem*.

K = killed. D = died. G = 30 green shoots fed *per diem*.

C.L.O. = 2 drops of cod-liver oil replaced shoots.

As the result of such treatment growth was restored in all four cases, demonstrating beyond doubt the activity of the etiolated wheat shoots in repairing deficiency of vitamin A. At the time of the adoption of the revised routine, however, only two animals of the control group remained alive. Two more rats were therefore placed on the basal diet, receiving in addition an allowance of thirty wheat seeds each per day and direct irradiation from the commencement of the experiment. After the normal period they declined and died, one displaying severe symptoms of xerophthalmia.

Discussion.

Since feeding was conducted after nightfall the shoots were exposed only to artificial illumination before ingestion by the rats. It seems improbable that illumination of such feeble intensity should cause a formation of the vitamin. The rats themselves, however, were not kept in the dark, and a loophole for photosynthetic action remains open if the formation of the vitamin, as in the case of vitamin D, be pictured to take place in the animal on exposure to diffuse daylight or to mercury arc illumination. Further experiments are contemplated to clear up this point. For the present there is no reason to believe that vitamin A is not produced by the plant in the dark. If it is not formed under such conditions a precursor must at least make its appearance, since wheat seeds fed under similar conditions of illumination were found to be ineffective.

From the growth curves it will be seen that for a period of several weeks all the rats receiving etiolated shoots grew at an approximately normal rate. In the case of rat no. 1 (male) such growth was continued without serious check for about four months, a weight of 172 g. being attained 115 days after the addition of shoots to the diet. The remaining rats (nos. 2, 3 and 4 (females)) after a time ceased to grow, marked plateau periods in the growth curves being attained at body weights of about 130 g. The cause of this stoppage is not known. Although no acute symptoms, beyond occasional sneezing, were observed, all the four rats were obviously in an imperfect state of nutrition, rib bones being unduly prominent, coats slightly rough, backs slightly arched and muscular tone poor. A partial insufficiency of the vitamin might well be suspected, but shoots were fed at six times the minimum determined by Coward; moreover the replacement of etiolated shoots successively by green shoots and by cod-liver oil in the diets of two of the rats, while promoting slight growth, did not notably improve their condition above that of the others. Whatever the cause of such imperfect nutrition may have been, however, it would appear certain that the etiolated shoots, directly or indirectly, provided an active source of vitamin A, since the rats could certainly not have survived the control animals for several months without any of the characteristic signs of deficiency in the continued absence of that vitamin.

In the present experiments, it should be observed, shoots at an advanced stage of development were employed. In experiments with wheat and corn seeds germinated for shorter periods (20–48 hours), Coward [1927], Harrow and Krasnow [1922, 1924], and Karshan, Harrow and Krasnow [1927] have failed to establish any increase in vitamin A content above that of ungerminated seeds, and it has been suggested that the vitamin is not synthesised under such conditions. In Coward's experiments, however, only five germinated wheat seeds were allowed per rat per day, a level dangerously low in view of past experiences at a three-shoot dosage, while in those of the American workers vitamin D was not supplied. Such evidence, therefore, while of obvious importance from an economic standpoint, cannot afford an adequate basis for the absolute denial of the formation of traces of vitamin A, and for the present the question should be left open. Stepp [1925], it may be mentioned, has reported the synthesis of traces of vitamin A in wheat seeds germinated for 4 days, but as no precautions were taken to exclude the action of light this finding cannot be considered relevant to the problem under discussion.

SUMMARY.

Fresh etiolated wheat shoots, 10 days old, fed to rats receiving a diet deficient in vitamin A, but adequate in vitamin D, at the rate of thirty shoots per day (equivalent to 1·5 g. of dry wheat) prevented death and restored growth. Seeds fed at the same rate were ineffective.

This result is in agreement with statements by Wilson [1922] and Coward [1927] that vitamin A may be formed in etiolated tissues. In the present experiments the feeding of shoots was continued for some months after the death of the control animals. The final condition of the animals was fair, although they appeared somewhat lean and rough-coated when compared with similar rats receiving a liberal diet.

My thanks are due to Sir F. G. Hopkins for support and criticism.

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CXIX. THE INFLUENCE OF TEMPERATURE ON THE NATURE OF THE FAT FORMED BY LIVING ORGANISMS.

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(*Received July 5th, 1927.*)

Leathes and Raper [1925, p. 119] have put forward the view that the temperature at which fats are formed in nature is one of the factors which determine their degree of unsaturation. This suggestion is based mainly on three lines of reasoning. Firstly, a general survey of the fats found in animals and plants indicates that the highly unsaturated fats are most commonly found in plants growing in temperate or cold regions and in the poikilothermic animals which live under relatively cold conditions, whilst the most saturated fats are found in tropical or subtropical plants and in warm-blooded animals. The carnivora should not be included in this generalisation because the composition of their adipose tissue fat is partly, at any rate, determined by the fat which they take as food, and even in animals such as the pig, which produce fat easily from carbohydrate, differences have been observed in the composition of their stored fat which may be referred to differences in the composition of the fats present in the cereals on which they have been fed. Secondly, it is usual to find that the fat present in living organisms is fluid at the temperature at which the organisms exist. This would preclude the formation of saturated acids to any great extent in organisms living under temperate or cold conditions because such acids would tend to raise the melting points even of mixed glycerides which contained them. The higher melting point of the saturated acids would be less important from this point of view in the warm-blooded animals or tropical plants and it might be more likely therefore to expect saturated acids to occur to a greater extent in organisms living under warmer conditions. Thirdly, various hypotheses have been made regarding the chemical reactions by which fatty acids are formed in nature, *viz.* autocondensation of acetaldehyde [Leathes, 1906], condensation of pyruvic acid and acetaldehyde [Smedley and Lubrzynska, 1913], or autocondensation of pyruvic acid [Neuberg and Arinstein, 1921]. All these admit of the possibility of the formation of long chains of carbon atoms with unsaturated linkages as products of the condensation reactions which produce them and if they are to give rise to saturated carbon chains a reduction must take place subsequently.

If this represents what takes place, then the unsaturated acids would be the precursors of the saturated. Two different types of reaction are involved, namely, condensation and reduction. It seems possible that both these reactions would not necessarily take place with the same ease at low temperatures and, if it be assumed that the condensation takes place readily at low temperatures whereas the reduction requires a higher temperature, an explanation of the distribution in nature of the saturated and unsaturated acids referred to above might be found on these lines. This would not imply, as might at first sight be thought, that at any given temperature the exact degree of unsaturation of a fat would be determined solely by that temperature. It would also depend on other factors, *e.g.* the amount of the catalyst present which brings about the reduction, or the extent to which the synthesis into glycerides was taking place, thus possibly inhibiting further reduction.

It seemed to us that it would be possible to test the main hypothesis as to the influence of temperature, experimentally, by growing an organism at different temperatures, all other conditions being kept the same. Certain of the fungi seemed likely to be suitable subjects since they grow fairly readily at different temperatures and some of them contain notable amounts of fat. The work was actually begun three years ago but, owing to one of us (L. K. P.) having to withdraw owing to unforeseen circumstances, it had to be discontinued in 1926 and a further opportunity to continue it has not yet arisen. In the meantime, Terroine, Bonnet, Kopp and Véchot [1927] working on almost the same lines have obtained results in complete accord with our own. We have therefore thought it advisable to publish the data we have obtained up to the present. The work will be extended when a suitable opportunity offers.

EXPERIMENTAL.

Two fungi were used, namely, *Aspergillus niger* and *Rhizopus nigricans*. The former was grown on Czapek's solution to which 1 % ammonium sulphate and 3 % glucose were added. The latter would not grow on this medium but grew well in Meyer's solution with the addition of 1 % asparagine and 3 % glucose. The fungi were grown in litre flasks containing 500 cc. of the medium which were sterilised in the usual way. It was found possible to grow *Aspergillus niger* at three temperatures, namely, 18°, 25° and 35°, whereas *Rhizopus nigricans* was grown only at 12° and 25°, it being found impossible to grow this organism at 35°. The rate of growth varied with the temperature and it was allowed to continue till a good mat of the mould covered the surface of the medium. In some instances the fungus was left for some time after this had occurred in order to see what effect this would have on the amount of fat present and on its iodine value.

The methods used for extracting the fat from the mould were as follows. The mat of fungus was broken up, strained and pressed on a Büchner funnel and well washed with distilled water; after roughly drying between filter-paper it was weighed. It was next covered with alcohol in a wide-necked flask and allowed to stand at least 24 hours. The alcohol was then removed by filtration and the fungus extracted with hot alcohol by suspending it in a thimble from the lower end of a reflux condenser which was attached to a flask containing 75 cc. boiling absolute alcohol. After extracting for 3 hours, the alcohol was removed from the flask and a further extraction with a fresh portion of 75 cc. alcohol was carried out. The three alcoholic extracts were combined, 15 cc. of 50 % aqueous KOH were added and most of the alcohol was distilled off on the water-bath, the fat undergoing saponification during this process. The fatty acids and unsaponifiable matter soluble in light petroleum were obtained from this solution by acidification and shaking with a measured quantity of light petroleum as in the usual method of fat estimation by the modified Liebermann method [cf. Leathes and Raper, 1925, p. 58]. From an aliquot part of the light petroleum extract, the fatty acids were removed by shaking with dilute sodium hydroxide in 50 % alcohol and then, after liberating the acids again with sulphuric acid, they were taken up in light petroleum, the solvent was distilled off in an atmosphere of CO_2, the flask finally evacuated for half an hour whilst in a boiling water-bath and the fatty acids weighed. The mean molecular weight was determined by titration in excess of alcohol with aqueous $N/10$ NaOH and, after recovery of the fatty acids from this solution by acidifying and extracting with light petroleum, their iodine value was determined by Wijs' method.

The results obtained are given in the accompanying table.

Fungus	Temp.	Time of growth (days)	Moist wt. of fungus (g.)	Total wt. of fatty acids (g.)	Mean molecular wt.	Iodine number	Mean iodine number
Aspergillus niger	18°	17	49·8	0·323	303	146·7	149
		17	46·3	0·206	300	153·6	
		56	39·9	0·084	323	150·2	
		56	41·5	0·086	330	145·9	
	25°	10	59·4	0·276	287	124	129
		14	50·3	0·302	304	132·5	
		17	51·1	0·254	290	131·3	
		35	40·5	0·252	287	127	
	35°	6	39·2	0·468	293	92·1	95
		7	38·8	0·527	285	92·3	
		9	37·8	0·633	290	99·9	
Rhizopus nigricans	12°	30	34·6	1·705	281	88·7	88
		30	31·0	1·669	289	87·2	
	25°	10	32·5	1·082	288	79	78
		13	35·4	1·168	287	77·3	

DISCUSSION.

It will be seen, with both fungi, that the more unsaturated fats are produced at the lower temperatures, and, in the case of *Aspergillus niger*, the fat produced at a temperature intermediate between the highest and lowest had also an intermediate iodine value. The period of growth does not appear to affect the results at any given temperature. Comparable results have been obtained by Terroine, Bonnet, Kopp and Véchot [1927] with the mould *Sterigmatocystis nigra*[1]. When grown at 35° the iodine value of its fatty acids was 85, whereas when grown at 17° it was 114. With the Timothy Grass bacillus the iodine value of the fatty acids was 33 when grown at 35° and 58 at 14°. These observers also determined the iodine value of the total fatty acids of a mollusc (*Melanopsis donneti*) and a fish (*Astatotilapia desfontainesi*) living at a temperature of 30°, the figures obtained being 91 and 87 respectively, whereas the fatty acids of a mollusc (*Limnaea peregra*) living at 8° had an iodine value of 125. These results all support the view that the temperature of the environment has an influence on the degree of unsaturation of the fatty acids which organisms produce. This however does not prove that all the deductions are correct which led to the hypothesis that temperature influences the nature of the fat formed in living organisms. Although in our own work, and that of Terroine and his colleagues, temperature was the only variable imposed by the experimenters on the different organisms, it was not necessarily the only variable in the experiments. It has been shown by the French workers that the composition of the organisms grown at different temperatures is not absolutely the same, though the differences are not very great. Then, difference in conditions of growth, *e.g.* temperature, the rate of growth, the type of medium, the exhaustion of the medium, affect the morphological character of fungi and have some influence on spore formation. These modifications of the life processes of the organisms imply that the chemical reactions which are normally taking place in them will be considerably modified. For these reasons it cannot be considered that the few experiments which have been carried out in this field of work prove that the changes observed in the composition of the fatty acids in organisms grown at different temperatures are simply the result of the effect of temperature on the two chemical reactions which have been thought to play a great part in fat formation, *i.e.* condensation of some simple aliphatic substance and reduction of the unsaturated compounds produced. Terroine and his colleagues, by comparing the energy loss from the medium during growth with the potential energy of the fungus formed at different temperatures, have shown that the expenditure of energy is somewhat greater at the higher temperatures. This indicates that the production of the more saturated acids under these conditions involves a greater utilisation of the potential energy of the medium and suggests that reduction succeeds condensation in the series of reactions by which fatty acids are produced, and thereby involves a greater expenditure of energy.

SUMMARY.

1. The iodine value of the fatty acids produced by *Aspergillus niger* and *Rhizopus nigricans* varies with the temperature at which these organisms are grown.

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[1] A synonym of *Aspergillus niger*.

2. At higher temperatures the fatty acids produced are more saturated, this being in conformity with the observation that, in general, the more saturated fats found in nature are usually produced at higher temperatures than the less saturated ones.

We desire to express our thanks to Professor Wilfred Robinson for pure cultures of the fungi used and to the Government Grant Committee of the Royal Society for a grant in aid of the expenses of this investigation.

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CXX. THE GLUCOSE METABOLISM OF KIDNEY TISSUE *IN VITRO*. I.

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(*Received July 7th, 1927.*)

Recent work on the relationship between glycolysis and respiration in normal tissues by Warburg and Meyerhof and their associates has shown that the changes previously found to take place in muscle, occur in most body cells. The experiments to be described below have been undertaken partly to determine more fully the part glucose takes in these changes, and also to see if the theory of surface activity previously adumbrated by me [1926] for blood corpuscles applies to other body cells. So far only the kidney has been investigated.

Experimental.

In all the experiments undertaken, fresh rabbit's kidneys were employed. They were perfused with warm saline till free from blood and the cortex was then cut off with small scissors and finely chopped with a razor. Weighed amounts of this were then placed in isotonic phosphate buffer at a p_H of about 8·0. The present writer has found that the best proportions of kidney tissue to phosphate buffer are one part of the former to four of the latter, and this proportion has been used throughout. Enough glucose (Kahlbaum) was added to bring the final concentration up to from 0·10 % to 0·20 %.

Sugar estimations were carried out on the supernatant fluid by the method of Hagedorn and Jensen. In the absence of glucose the reducing power did not change, or rose very slightly, while the distribution of glucose between buffer and kidney appeared to reach an equilibrium almost immediately. It thus appeared justifiable to attribute changes in reducing power to alterations in glucose content.

The tubes containing the suspension were completely immersed in a waterbath at 38° and were continually shaken by a mechanical device so that the contents were thoroughly mixed during the entire time of the experiment.

In several of the experiments, hydroxyquinoline sulphate was used in a concentration of 1 in 1500 as an antiseptic. Control experiments, in which the kidney tissue was removed from a suspension after incubation for 4 hours at 38°, and the phosphate-glucose solution reincubated, showed that the glucose content did not change subsequently. In addition, infection was not at all probable during the short period the experiments lasted, which seldom exceeded 4 hours, and in the majority of cases was no longer than two.

FACTORS GOVERNING THE RATE OF GLUCOSE UTILISATION.

Immediately incubation was begun, the glucose content of the phosphate buffer was found to fall at a rate which was fairly constant for each specimen, but varied somewhat from kidney to kidney, the amount used % per hour being between 0·015 and 0·040 g. Exp. 1 in Table I and Fig. 1, curve 1, show results typical of many obtained. The value of *k* as calculated for a monomolecular reaction rises as the reaction proceeds, which would suggest that the rate was linear. The power of utilising glucose was found to be lost or greatly weakened if the kidney was kept for 24 hours at 0°.

In certain experiments, however, discrepant results were obtained which suggested that other factors might be playing a part. A series of experiments was undertaken in which the suspensions were incubated *in vacuo* in Thunberg tubes. The change in reducing power was estimated during this period, and the suspension was then placed in an aerobic tube and reincubated, and changes in the reducing power noted. Typical results of these experiments are shown in Table I, Exps. 2, 3 and 4, and Fig. 1, curve 2.

Table I.

Exp. no.	Conditions	Hours	Glucose %	Glucose utilised %	*k*
1	Aerobic	0	0·179	—	—
	,,	1	0·154	0·025	0·0645
	,,	2	0·129	0·050	0·0715
	,,	3	0·110	0·069	0·0707
	,,	4	0·092	0·087	0·0725
2	Anaerobic	0	0·199	—	—
	,,	2	0·202	0	—
	Aerobic	0	0·202	—	—
	,,	1	0·173	0·029	—
	,,	2	0·155	0·047	—
	,,	3	0·148	0·054	—
3	Anaerobic	0	0·226	—	—
	,,	2	0·217	0·009	—
	Aerobic	0	0·219	—	—
	,,	1	0·199	0·029	—
	,,	2	0·186	0·042	—
4	Anaerobic	0	0·195	—	—
	,,	1·5	0·195	0	—
	Aerobic	0	0·191	—	—
	,,	1	0·170	0·021	—
	,,	2	0·161	0·030	—

These figures show that the presence or absence of oxygen plays an important part in the utilisation of glucose, and that this can be completely stopped *in vacuo*, while, during the subsequent aerobiosis, glucose is again utilised. For these results to be consistently obtained, it is essential that the kidney be first thoroughly freed from blood, as otherwise enough oxygen is held by the haemoglobin remaining to promote some glucose utilisation.

The utilisation of glucose was also found to be markedly inhibited by cyanide. Experiments were undertaken using KCN in a final concentration of *M*/500 and *M*/250. Before being used, the cyanide solution was adjusted to the same p_H as the phosphate buffer. Control experiments with KCN and kidney tissue in the absence of glucose showed that the reducing power did not change, and it was also shown that KCN did not interfere with the sugar estimations.

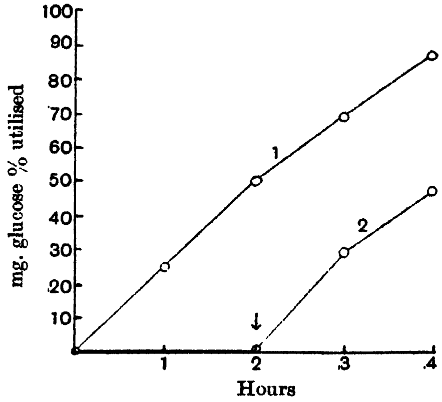

Fig. 1. The rate of utilisation of glucose.

Curve 1. Aerobic incubation.
Curve 2. Anaerobic and aerobic incubation. At the arrow, the suspension was transferred from an anaerobic to an aerobic tube.

Typical results are shown in Table II.

Table II.

		Control tube		Experimental tube		
Exp. no.	Hours	Glucose %	Glucose utilised %	Glucose %	Glucose utilised %	Concentration of KCN
1	0	0·157	—	0·163	—	*M*/500
	1	0·195	0·062	0·163	0	
	2	0·059	0·098	0·161	0·002	
	0	—	—	0·125	—	*M*/250
	1	—	—	0·129	0	
	2	—	—	0·131	0	
2	0	0·139	—	0·139	—	*M*/250
	1	0·106	0·033	0·132	0·007	
	2	0·081	0·058	0·125	0·014	
3	0	0·181	—	0·163	—	*M*/500
	1	0·145	0·036	0·159	0·004	
	2	0·119	0·062	0·173	0	
4	0	0·138	—	0·129	—	*M*/250
	1	0·104	0·034	0·129	0	
	2	0·079	0·059	0·129	0	
	0	—	—	0·139	—	*M*/250*
	2	—	—	0·129	0·010	

* 0·04 mg. Fe added (as used by Harrison [1924]).

In Exp. 4 iron was added as ferrous ammonium sulphate. This experiment, which is typical of several, shows that added iron does not influence the inhibition by cyanide. It has also been found that KCN inhibits the glucose utilisation by unperfused kidneys to the same extent as by perfused, which would tend to show that iron is not acting catalytically.

The possibility cannot be disregarded that during these experiments in which no glucose appears to be utilised, an equal amount of other reducing bodies may be formed. Even if this were so, it would still show that glucose is differently utilised aerobically and anaerobically. The constancy of the figures obtained at the beginning and end of anaerobiosis appears to make it more probable, however, that glucose is not being utilised during this period.

Levene and Meyer [1913] showed that chopped kidney tissue *in vitro* was capable of turning glucose into lactic acid, an observation which has also been recorded by Warburg, Posener and Negelein [1924]. The latter writers found, however, that the rate of glycolysis was greater anaerobically than aerobically, a fact which does not at first sight accord with the findings recorded above, but it is possible that different phases of carbohydrate utilisation have been studied in the two cases.

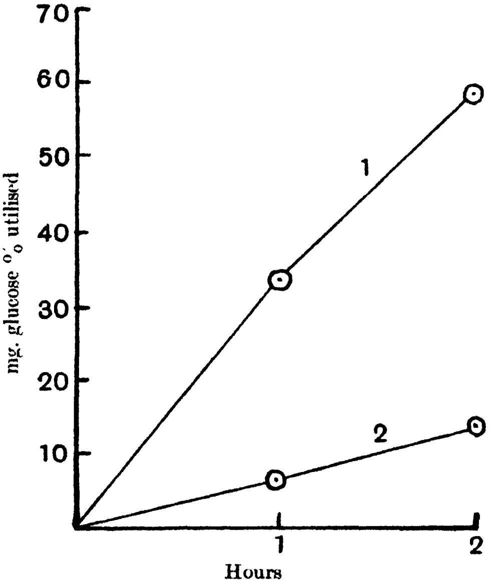

Fig. 2. The influence of cyanide.

Curve 1. Normal glucose utilisation.
Curve 2. Glucose utilisation in the presence of KCN *M*/250.

A few preliminary experiments have shown that the disappearing glucose may be converted into a polysaccharide[1]. In addition, it has been found that the contents of the reaction tubes are more acid at the end of the experiments than at the beginning, irrespective of whether glucose has been broken down or not. This fact would make it appear more probable that glucose was taking part in the synthetic phase of the glycogen $\rightleftharpoons$ lactic acid cycle as found by Meyerhof, Lohmann and Meier [1925], particularly as Meyerhof [1926, 2] has shown that glucose is probably not an intermediate in the production of lactic acid from glycogen in muscle. Further work is being done to determine the fate of the glucose which is utilised.

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[1] Note added August 3rd. More recent experiments have shown that it is improbable that glucose is changed to polysaccharides by kidney tissue *in vitro*. J. T. I.

The method of utilisation of glucose in the kidney appears, however, to differ fundamentally from that in the blood corpuscle, since the latter is not inhibited by cyanide [Irving, 1926].

THE SITE OF GLUCOSE UTILISATION.

A series of experiments was undertaken with a view to finding the nature of the agency which activated glucose. Attempts were first made to isolate a "glycolytic" enzyme by extraction, employing a method similar to that used by Meyerhof [1926, 1].

The extracts were usually made by grinding the chopped cortex with isotonic phosphate buffer (p_H 7·8) at $-3°$. Both the whole mass and the supernatant fluid after centrifuging off the macerated tissue were quite inactive when incubated at 38°. In addition, at the end of the ordinary aerobic experiments described in the previous section, the phosphate buffer, after removal of the kidney tissue, was not found to utilise glucose, so that the enzyme did not appear to be able to diffuse out of the cells. Finally, kidney tissue which had been previously frozen solid for a short period, and then thawed, was found to have completely lost its activity, an observation which Laquer [1922] has also made for muscle.

Alders, Chiari and Laszlo [1927] have recently stated that a glycolytic enzyme can be prepared from several tissues by incubating them in glucose-Ringer solution for 1 hour, and then filtering through a Chamberland candle. The filtrate possesses the power of degrading glucose. The present writer has not succeeded as yet in confirming these experiments, but the method of analysis employed by these authors appears to include other substances besides glucose.

It would thus appear that if an enzyme were responsible for the changes observed, it would be much less stable than that described by Meyerhof [1926, 1]. Further experiments were carried out in order to see if an active surface was involved. Several homologous alcohols, methyl, ethyl, propyl, and *iso*propyl were employed. The minimum inhibiting concentrations were investigated in each case, and were found to be very sharp, save in the case of *iso*propyl alcohol, which for some unknown reason did not give such good results as the others. These concentrations were:

Methyl alcohol...	9·9 %
Ethyl alcohol	7·8
Propyl alcohol...	2·9
*iso*Propyl alcohol	1·5

The ratio between these amounts is consonant with a surface action theory. Typical results are shown in Table III.

Table III.

Alcohol %	mg. glucose % utilised in 2 hours		
	Propyl	Ethyl	Methyl
1·5	39	—	—
2·0	9	—	—
2·4	7	—	—
2·9	0	—	—
3·4	0	—	—
3·8	—	14	—
5·7	—	—	23
6·5	—	10	—
7·1	—	18	—
7·4	—	7	22
7·8	—	0	—
8·3	—	0	—
9·1	—	—	9
9·9	—	—	0
10·7	—	—	0

In addition, reversibility of the inhibiting action was demonstrated during the early stages of the experiments. Typical results are shown in Table IV.

Table IV.

In each experiment sample no. 1 was placed in contact with alcohol for the stated period, the kidney tissue was washed in a large excess of saline and then incubated in glucose-buffer solution. Sample no. 2 was incubated in glucose-buffer with the same concentration of alcohol as a control.

		Sample no. 1		Sample no. 2		
Exp. no.	Hours	Glucose %	Glucose utilised %	Glucose %	Glucose utilised %	Conditions
1	0	0·157	—	0·134	—	Propyl alcohol
	2	0·110	0·047	0·136	0	2·9 %. 10 mins.
2	0	0·141	—	0·110	—	Ethyl alcohol
	2	0·090	0·051	0·120	0	7·8 %. 5 mins.
3	0	0·163	-	0·141	—	Methyl alcohol
	2	0·127	0·036	0·141	0	9·9 %. 10 mins.

These experiments thus show that an active surface is probably one of the main factors in the utilisation of glucose by the kidney. Whether the surface acts in the manner pictured by Quastel [1926], or whether the responsible enzyme is adsorbed on some cellular interface, it is at present impossible to say. From the facts adduced above, however, it would appear that, as with the blood corpuscle, the integrity of the cell is essential for the utilisation of glucose.

SUMMARY.

1. Kidney tissue *in vitro* utilises glucose aerobically at a rate which is probably linear. *In vacuo*, or in the presence of KCN, the utilisation of glucose is markedly inhibited, or stopped completely.

2. The integrity of the kidney cell is essential for the utilisation of glucose. It is probable that an active surface is involved.

The writer is indebted to Prof. R. A. Peters for his continued advice and interest in this work.

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CXXI. VITAMINS A AND D OF SPINACH.

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Recent work on the differentiation of vitamins A and D has emphasised the need of a vitamin D-free source of vitamin A. This has been chiefly sought for in fresh spinach leaves, which have been shown by many investigators to contain considerable amounts of vitamin A. However, in spite of much work on the problem, the possible presence of vitamin D in spinach leaves still appears to be a matter of debate.

The first investigators to use spinach for the supply of fat-soluble vitamins appear to have been Steenbock and Gross [1920], who found that the dried leaves when fed to rats at the level of 5 % of the diet as the sole source of vitamin A, produced continued but subnormal growth over periods of 6 or 7 months. Osborne and Mendel [1920], employing an ether extract of dried spinach leaves, obtained adequate growth and cure of xerophthalmia with doses of 66 mg., equivalent to about 10 to 20 g. of fresh leaves. McClendon and Sedgwick [1921] secured satisfactory growth in rats with dried spinach administered in amounts equivalent to about 5 or 6 g. of the fresh leaves, and also found it of benefit for children. In these investigations vitamin A was not distinguished from vitamin D.

After the differentiation of the fat-soluble vitamins attention was turned to the possible antirachitic properties of spinach. McClendon and Shuck [1923], using dried spinach in amounts equivalent to about 8 or 9 g. of the fresh leaves, observed no antirachitic action. Zucker and Barnett [1923], who employed an acetone-ether-alcohol extract of the dried leaves, found its growth-promoting potency comparable with that of cod-liver oil, but no significant amount of vitamin D was detected. The amount fed, however, was not stated. Goldblatt and Zilva [1923], feeding the fresh leaves, obtained some growth with less than 0·25 g., but no increase in calcification in rats on a rachitogenic diet with doses up to 3 g., as much as it was found practicable to administer, and concluded that "there is a great disparity between the growth-promoting and the antirachitic potency of spinach."

The next step came when it was discovered that the antirachitic vitamin could be formed by the action of ultra-violet light, either that naturally present in sunlight, or artificially produced. Hess and Weinstock [1924] found no appreciable amount of vitamin D in spinach or other green leaves, but were able to produce it by irradiation. Following up this idea, Chick and Roscoe [1926] showed that fresh spinach might contain slight amounts of vitamin D if collected in the summer, while Boas [1926] concluded that winter spinach contains an amount of vitamin D which is negligible compared with its content of vitamin A. Later, an attempt was made by Roscoe [1927] to increase the content of vitamin D by further exposure of the leaves to the ultra-violet rays in sunlight, but the results obtained were not conclusive. Thinking that this might be due to rats not eating sufficient greenstuff, Roscoe also tested "sunned" spinach on rabbits. It could not be shown conclusively that exposure to sunlight increased the content of vitamin D, but Roscoe succeeded in demonstrating that fresh spinach contains more of this vitamin than had previously been suspected.

While the substitution of the rabbit for the rat as an experimental animal in testing for vitamin D enables larger proportions of spinach to be included in the diet, our opinion was that the use of this modified technique involves new difficulties. First, the digestive and assimilative processes in a herbivorous animal are different from those in an omnivorous animal, the gastro-intestinal p_H varying considerably and being closely connected with the rôle of the vitamin [Redman, Willimott and Wokes, 1927]. Secondly, from the recent work of Mellanby and Killick [1926], it seems probable that the optimum conditions for calcium absorption in the rat differ from those in the rabbit. Thirdly, the administration of large doses of fresh spinach leaves necessarily involves the ingestion of considerable quantities of calcium and phosphorus, with consequent alteration of the calcium/phosphorus ratio. (Fresh spinach contains 0·09 % CaO and 0·13 % P_2O_5 according to Sherman [1911].) For these reasons we decided to employ a practically mineral-free extract of dried spinach leaves which could be administered to rats in large doses without upsetting the calcium/phosphorus ratio.

EXPERIMENTAL.

Preparation of the spinach extract.

7167 g. of fresh English spring spinach (picked at the beginning of May, 1926) were sorted, spread out on sheets of paper and dried at 18° for 2 days. The resulting product was a light green material weighing 726 g. This was first broken up by rubbing between the hands, the midribs and stalks were then removed, and the residue was ground in a mortar and sieved through a no. 10 sieve. The product was a light green powder weighing 640 g. This material was shaken with dry ether at room temperature for 3-hour periods, three successive lots of about 3 litres each being used. The first extract was dark green leaving a residue coloured light yellow-green. The marc was pressed on a Büchner funnel by hand so as to remove as much of the solvent as possible. This pressed marc on treatment with a second lot of ether gave a much less darkly coloured extract, while the third extraction yielded a product only slightly coloured. The residue was then extracted with two successive lots of dry acetone, but little colour was obtained. The whole of the ether and acetone extracts were filtered, combined, and the solvent removed as rapidly as possible *in vacuo*. The resulting product was a dark green pasty mass, weighing 32·5 g. and representing 0·45 % of the fresh leaves. This was put aside in a desiccator protected from light, over sulphuric acid, and after a few days small red crystals were observed, probably of carotene and xanthophyll. The extract was stored in a desiccator filled with nitrogen and protected from light while in use.

Tests applied to the extract showed it to be free from oxidising enzymes (oxygenase and peroxidase). The ash was about 4·5 %. Since the fresh leaves contain about 2 % of ash [Plimmer, 1921], and the extract was concentrated over 200 times, only about 1 % of the mineral matter in the fresh leaves had passed into the extract. On taking up in light petroleum a few grams of the extract, decolorising with norite, filtering and evaporating, a colourless oily residue was obtained which gave transient blue colours with both arsenic trichloride and antimony trichloride [Willimott and Wokes, 1927]. Takahashi [Takahashi *et al.* 1925] using a vitamin A concentrate prepared both from cod-liver oil and from spinach, obtained transient blue colours with concentrated sulphuric acid, but we have not been able to trace any record of these colours, supposedly due to the vitamin, having been previously obtained with the former two reagents on a spinach extract.

Feeding experiments using high fat diets.

In a previous communication [Redman, Willimott and Wokes, 1927] it was pointed out that in tests for vitamin D two types of diet have been principally used by different investigators, viz. a high fat diet containing 15 to 17 % of fat, and a low fat diet containing less than 3 %. The high fat diet resembles the natural diet of the infant the more closely both in fat content and in its calcium/phosphorus ratio. It has been largely used by English workers, who have adopted bone changes as their chief criteria of antirachitic potency, but so far as we are aware, no results have yet been published of the faecal p_H of rats on high fat diets free from vitamin D. In view of the findings of Zucker and Barnett [1923] and the more recent work of Jephcott and Bacharach [1926] that the faecal p_H in the rat can be used to test for vitamin D, we thought it of interest to feed to rats a high fat diet in which the spinach extract previously described served as sole source of vitamin A, and vitamin D if any. The faecal p_H was observed as well as the bone changes more commonly employed as criteria for vitamin D.

Standard albino rats of Wistar Institute ancestry were placed on the basal diet usual in this laboratory containing 15 % fat, vitamin B being supplied by an extract of yeast and vitamin C by lemon juice. The spinach extract was administered at first by hand in advance of the basal ration but as the animals consumed it readily without objection, the required dose was afterwards uniformly mixed in with the basal diet. By following the food intake, it was possible to give an amount of the extract such that each animal received on the average about 25 mg. Much larger amounts are consumed by rats without difficulty.

Table I. *Spinach extract used with a high fat diet as sole source of vitamins A and D.*

Group	Sex	No. of rats	Supply of vitamins	Daily dose (mg.)	Average weekly weights (g.)	Rickets *		Xerophthalmia
						Microscopic	A.R. ratio	
Experimental 1	♂	3	Spinach extract	25	55 72 79 91 102 109 126 130 133 141	Very slight	1·15	Absent
	♀	3	Spinach extract	25	48 60 74 82 87 95 98 100 101 106	Very slight	1·21	Absent
Positive control	♂	3	Cod-liver oil	50	56 72 94 119 141 164 178 194 210 221	Absent	1·25	Absent
	♀	3	Cod-liver oil	50	48 62 82 96 107 118 129 138 142 143	Absent	1·29	Absent
Negative control	♂	2	Vitamin D as direct irradiation of the animals	—	55 59 72 85 88 87 84 86 75	Absent	—	Present in two animals
	♀	3		—	53 55 68 80 81 83 83 76 74	Absent	—	
					Average faecal p_H			
Experimental 1	♂ & ♀	6	—	—	6·8 7·0 7·1 7·4 7·4 7·4 7·5 7·5 7·5	—	—	—
Negative control	♂ & ♀	5	—	—	6·8 7·0 6·9 6·8 6·7 6·8 6·7 6·8	—	—	—

On account of the different rates of growth in the two sexes, separate records were made of the average weights of males and females, in each of the three groups. The groups were chosen so as to be well balanced in regard to age, weight, sex and litter origin, each containing originally three males and three females, but in the negative control group one male died just after the beginning of the experiment from some unknown cause, and was not included in the average data. Of the female negative controls, one died in the third week and a second in the eighth week, and their weights are included in the average data. The surviving female, and the two males, were chloroformed and autopsied at the end of 8 weeks, when vitamin A deficiency was pronounced.

The experimental results are summarised in Table I. It will be seen that 25 mg. of the spinach extract, equivalent to about 6 g. of the fresh leaves, secured freedom from xerophthalmia and steady though subnormal growth in all the six experimental animals over a period of 10 weeks. It is noteworthy that Chick and Roscoe [1926], using a similar high fat diet, and fresh spring spinach, did not succeed in entirely preventing xerophthalmia with doses reaching in one case as high as 2·6 g., although the growth obtained was rather better.

In regard to rickets, the bone changes in our experiments were not conclusive. Autopsies on the animals revealed very slight rickets in several but not in all cases, which is in agreement with the findings of Chick and Roscoe. The average A.R. ratio was only very slightly lower than normal. On the other hand, the faecal p_H became alkaline and, while fluctuating somewhat, remained on the average faintly alkaline throughout the experiment. From this it would appear that an alkaline faecal reaction is specific to absence of vitamin D, whether rickets develops or not, but the matter requires further investigation. At the end of the experiment, vitamin D was supplied to the remaining animals in two ways, either as cod-liver oil or by direct irradiation of the animals, the diet not being altered, and in each case the faecal reaction changed within a few days to definitely acid (p_H below 6·8).

Feeding experiments using low fat diets.

The low fat, vitamin D-free diet has been used mainly by American investigators, who find that it produces within a few weeks a definite rachitic condition recognisable either by bone changes or by an alkaline faecal p_H. Since our previous experiments with the high fat diet had shown that a 25 mg. dose of the spinach extract was apparently too low to supply sufficient vitamin D, we decided to administer a much larger dose of 100 mg., equivalent to over 20 g. of fresh leaves, or more than twice as much as had previously been given by other workers testing for vitamin D in spinach.

Four standard albino rats were put on Zucker's modified low fat diet, consisting of patent flour 85, powdered egg-albumin 10, calcium lactate 2·8, sodium chloride 2, ferric citrate 0·2 parts by weight, with 2 g. of fresh spinach leaves per animal per day. The faecal p_H was ascertained daily, both colorimetrically and electrometrically, employing the methods previously described [Redman, Willimott and Wokes, 1927]. The results are shown in Fig. 1, in which the curve is built up from the average p_H of the total faeces collected from the group of animals. The individual variations between different animals, and between samples obtained from the same animal at different times on the same day, were investigated by means of the capillary electrode, and it was found that the greatest variation was 0·3 p_H, but on the average the results agreed to within 0·2 or less.

After 9 days on this diet, the faecal reaction had become definitely alkaline (average p_H 7·7). When it had remained alkaline for several days, each animal was given separately a daily dose of about 100 mg. of the spinach extract by hand, in advance of the basal ration, the supply of fresh spinach leaves then being discontinued. This was readily consumed without exception, and the food intake rose slightly from about 9 g. per head per day before dosing to about 11 g. after dosing. Xerophthalmia was absent throughout the experiment. A slight drop in alkalinity resulted on administration of the 100 mg. doses of extract, but at the end of another 12 days the faeces were still alkaline (average p_H 7·3, Fig. 1). On reducing the dose of extract to 50 mg., the faecal p_H became slightly more alkaline (average p_H 7·5). After the reaction had remained at this level for another 10 days, cod-liver oil was administered in doses of 0·1 g., and the change of average faecal p_H to below 6·8 within a week showed that the animals were reacting normally to the administration of vitamin D. The animals were immediately chloroformed and autopsied. Definite rickets was observed, and the A.R. ratio was found to range between 0·7 and 0·8. It would appear, therefore, that the spinach extract, when fed at a level supplying adequate amounts of vitamin A, does not contain significant amounts of vitamin D.

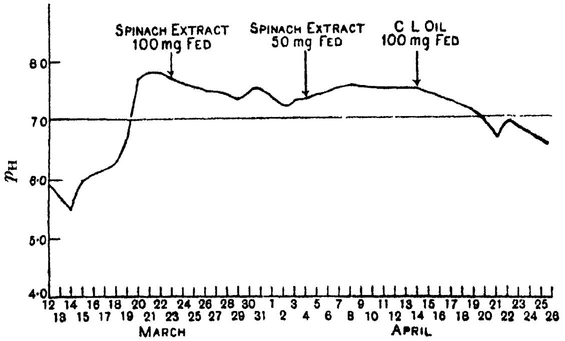

Fig. 1. Effect of feeding spinach extract to rats on Zucker's low fat rachitogenic diet.

Table II. *Summary of results obtained by various workers for content of vitamins A and D in spinach leaves.*

Reference	Type of diet	Form in which spinach was fed	Approx. equivalent in fresh leaves (g.)	Growth	Xerophthalmia	Rickets
Steenbock and Gross [1920]	—	Dried leaves	5–6	Steady but subnormal	Absent	—
Osborne and Mendel [1920]	High fat	Ether extract	15–20	Adequate	Cured	—
McClendon and Shuck [1923]	Low fat	Dried leaves	8–10	Adequate	Cured	No antirachitic property
Chick and Roscoe [1926]	High fat	Fresh leaves	0·5–2·6	Adequate	Very slight	Slight (? traces of vitamin D present)
Roscoe [1927]	Low fat	Fresh leaves	1·5	—	—	Severe, but small amount of vitamin D present
Willimott and Wokes [1927]	High fat	Ether-acetone extract	5	Steady but subnormal	Absent	Very slight
	Low fat	Ether-acetone extract	10	Adequate	Absent	Definite
	Low fat	Ether-acetone extract	20	Adequate	Absent	Some vitamin D present

NOTE. In calculating the approximate equivalent in fresh leaves of the doses of dried leaves, the fresh leaves have been assumed to contain about 92 % of moisture [Plimmer, 1921].

DISCUSSION.

In many previous investigations on spinach as a possible source of vitamins A and D, sufficient attention does not appear to have been paid to various factors which might affect the content of these two vitamins. Amongst these factors are exposure of the spinach to ultra-violet light, either artificially produced or naturally present in sunlight, to the action of oxidising enzymes known, from the work of Ciamician and Ravenna [1919], to be present in spinach leaves, or to heat and oxidation during the drying processes. Moreover, the variations in vitamin content of the fresh leaves, according to the amount of sunlight they have received, and the difficulty of feeding them regularly to rats in large doses, have thrown serious obstacles in the way of obtaining satisfactory quantitative data. In Table II are summarised results obtained by different workers, which show considerable variations in their estimate of the content of fat-soluble vitamins in spinach leaves. These differences are probably accounted for by the factors indicated above and possibly also by factors still unknown.

In our opinion, these varying factors can best be controlled by the use of an acetone-ether extract of the dried leaves which, when carefully prepared under given conditions, is a potent and stable source of vitamin A practically free from calcium and other mineral matter and from oxidising enzymes. The employment of such an extract has allowed the feeding of much larger doses of spinach than hitherto, and it has been found that the extract when fed at a level supplying ample quantities of vitamin A, does not contain more than traces of vitamin D. However, the fact that vitamin D may be present in these small amounts, which can be greatly increased by exposure to the action of the same agent (ultra-violet light) which under certain conditions appears to be responsible for the destruction of vitamin A, raises the interesting question of a possible balance between these two vitamins, a problem for further investigation.

SUMMARY.

1. An acetone-ether extract of dried spring spinach has been prepared more than 200 times as concentrated as the fresh leaves, and practically free from mineral matter and oxidising enzymes.

2. The extract was a potent and stable source of vitamin A in that 25 mg. daily doses secured complete freedom from xerophthalmia and induced satisfactory though slightly subnormal growth in rats.

3. The extract, when fed at this level, does not appear to contain significant amounts of vitamin D.

We are indebted to Sir F. G. Hopkins and to Prof. J. M. Beattie for their interest and support, and for kindly examining some of the experimental animals.

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CXXII. ON THE EARLY STAGES OF MICROBIOLOGICAL DECAY AND HUMIFICATION OF VEGETABLE TISSUES.

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(*Received June 4th, 1927.*)

In a previous communication [Thaysen, Bakes and Bunker, 1925] it was shown that the humification of vegetable tissues in peat bogs affects both the carbohydrate and the lignin constituents of the tissues. It was possible to demonstrate that the humus recoverable from peat is composed of two definite substances, one showing the characteristics of humus obtainable from carbohydrates after treatment with acid, and another functioning on chlorination as humus prepared from quinol or lignin.

A sample of humus behaving on chlorination as a typical carbohydrate humus was obtained also from a piece of ancient Egyptian linen cloth from the tomb of Tut-ankh-amen. The amount of humus collected from this source was of the order to justify the conclusion that it had been formed from the cellulose of the linen fibres through "ageing."

This was thought to indicate that the cellulose of vegetable tissues decaying in a peat bog might suffer a similar conversion through "ageing" and that the fraction of peat humus which had been shown to be identical with a carbohydrate humus in its behaviour on chlorination had been derived from cellulose.

In the course of certain investigations carried out on behalf of the Fuel Research Board, an opportunity arose of following the progress of decay of vegetable tissues, and it was thought of interest to ascertain the quantitative relationship of the two fractions previously recognised as present in natural humus, at various stages of the progress of humification, and to establish, if possible, the constituent of the vegetable tissues which served as raw material for the carbohydrate fraction.

The material which was subjected to humification was chopped oat straw. To secure humification its moisture content was increased to 50 % by addition of the requisite amount of water. In this water had been previously dissolved sufficient ammonium carbonate to raise the nitrogen content of the damped straw to 2 %.

After wetting, the straw was packed in boxes sufficiently large to hold 20 lbs. each. The sides of these boxes were made of wire netting to facilitate access of air to the material. The boxes were incubated at 22–25°. An inoculant was not added to the straw as previous experiments had shown that the microflora of oat straw is capable of producing the high temperatures which appear to be, if not essential, at least highly favourable for the humification to proceed rapidly.

Any loss of moisture suffered by the straw heaps was made good every third to fourth day.

The temperature of the packed straw rose rapidly and had reached a figure of 32° on the fifth day after incubation. On the seventh day a thermometer inserted in the heaps registered 49·6°. This temperature remained unchanged during the following 6 days and then gradually declined to 35°, a figure which was reached on the fortieth day. The lowest temperature recorded was 27·5° after 62 days' incubation of the straw.

A detailed bacteriological analysis of the decaying straw was carried out on the sixth day when the temperature of the straw had reached 46°. The number of micro-organisms developing on ordinary agar at 37° at this time amounted to 4900 millions per g. of damp straw, while 1800 millions developed on wort agar from the same quantity of straw. The microflora was very varied, though consisting essentially of aerobic bacilli of the groups of *Bac. mesentericus*, *Bac. dendroides* and *Bac. mycoides*. Various types of *Micrococcus* were observed and in addition *Bact. Zopfii*, *Bact. fluorescens liquefaciens*, *Bact. prodigiosum*, *Bact. herbicola aureum*, a number of actinomycetes and species of *Aspergillus* and *Penicillium*. The daily microscopic examination of the decaying straw revealed, as had already been observed by Miehe [1911], that the non-spore-forming types made their appearance first and were followed by various spore-formers when the temperatures had risen to a height.

On the twenty-fifth day an extensive growth of a species of *Coprinus* developed on the straw, the mycelium of which spread throughout the mass of the straw in the course of 2 days. Subsequently numerous fructifications of this fungus appeared on the surface of the straw.

By the forty-fourth day the microbiological activity appeared to have greatly diminished and most of the heat-resisting types could be observed only in the form of their spores. By inoculating dilutions of a suspension of the material into medium containing xylan and cellulose it was established that micro-organisms capable of decomposing both hemicelluloses and cellulose were present in the decaying straw.

There must have been more than 100 millions of hemicellulose-fermenting bacteria present per g. of straw since a dilution of 1 in 100 million of the straw showed growth in the xylan medium after 4 days' incubation at 37°. The types developing were not the aerobic spore-bearing rods capable of fermenting hemicelluloses (*Bac. mesentericus*, etc.).

The media containing cellulose which had been inoculated with 1×10^{-6} g. of decaying straw showed growth and destruction of the cellulose after incubation at 65°. Incubated at 37°, growth and cellulose decomposition did not occur from dilutions higher than 0·001 g. of straw. This observation indicates that a destruction of cellulose in the fermenting straw proceeded most vigorously from about the sixth to the twenty-ninth day, while the temperature of the straw remained above 37°.

The experiments by which it was hoped to throw light on the relative importance of the hemicelluloses and the cellulose for the formation of humus in decaying straw included the following determinations at repeated intervals.

(1) *The alkali solubility of the straw.* Here 4 g. of dried decaying straw were extracted under a reflux condenser with 200 cc. of 3 % sodium hydroxide. The residue was collected on weighed filter paper, washed, dried and weighed.

(2) *Hemicelluloses* (pentosans, estimated by Tollen's method [de Chalmot and Tollens, 1891; Günther *et al.*, 1890, 1891]).

(3) The *cellulose* (estimated by the method of Cross and Bevan [1903]).

(4) *Humic substances.* The filtrate from the alkali solubility determination was acidified with 30 cc. of concentrated hydrochloric acid, the precipitate left overnight and filtered through weighed paper, washed, dried and weighed.

The results of these determinations are compiled in Table I.

Table I*.

Sample	Days of fermentation before removal of sample	Temperature of heap on removal of sample ° C.	Alkali solubility of sample %	Hemicellulose content of sample %	Cellulose content of sample %	Humus content of sample† %
Original straw	--	25	48·5	31·3	39·86	22·4
Decaying straw	14	49·6	49·53	25·7	39·86	23·4
,,	28	40·5	56·66	18·97	35·94	26·26
,,	64	27·5	64·95	14·44	27·94	30·9
,,	82	27·5	67·84	11·07	23·78	31·55
,,	102	27·5	71·14	10·1	16·38	32·8
,,	146	27·5	70·6	7·93	—	32·3

* In interpreting the results of Table I it should be kept in mind that no attempt could be made to adjust the figures given for any losses suffered by the straw through the evolution of gases. The recorded data represent the percentages found in the sample as available on the day of collecting and after drying. The figures therefore under rather than over-estimate any changes which have occurred.

† It has been mentioned already that the figures given in this column were based on the weight of the precipitate collected on acidifying the extracts obtained by boiling the straw sample with a 3 % sodium hydroxide solution. They do not therefore give a true picture of the humus content of the samples since other substances, notably the hemicelluloses and lignin constituents, are brought into solution and reprecipitated under the conditions of experiment.

The most rapid increase in humus production took place between the first and the sixty-fourth day of fermentation, at a time when the hemicellulose content of the fermenting straw had decreased from 31·3 to 14·44 % and the cellulose from 39·86 to 27·94 %.

With a view to ascertaining the relative proportion of carbohydrate and lignin fractions in the humus during this and the immediately succeeding period, samples of humus were collected at various stages and subjected to chlorination by Eller's technique [Eller *et al.*, 1923].

The separation of the chlorinated fractions by extraction with ether showed, as indicated below (Table II), that the proportions of the two fractions varied appreciably with the progress of the humification.

Table II.

Days' fermentation before collection of humus from straw	Carbohydrate fraction in humus (%)	Lignin fraction in humus (%)
28	28·0	72·0
49	40·7	59·3
82	48·6	51·4
102	38·4	61·6
122	36·82	63·18

During the early period of humification of the oat-straw there was thus a steady rise in the percentage of the carbohydrate fraction, a rise which reached its maximum after about 80 to 85 days' fermentation. The extent of the subsequent decline in the percentage of this fraction could not be followed beyond the 122nd day of fermentation. However, it is interesting to note in this connection that a sample of silage which had remained packed in a silo for 3 years showed a humus content of 31·0 %, composed of 38·6 % carbohydrate humus and 61·4 % of lignin humus. And in a sample of peat humus examined previously [Thaysen *et al.*, 1925] and estimated to have taken 40 years to form, the carbohydrate fraction still amounted to 39·28 % of the total.

Whether the observed decrease in the lignin fraction of the humus was due to an actual destruction of lignin during decay or to an increase in the carbohydrate fraction beyond that indicated in Table II may be deduced from a consideration of the figures recorded in this and in Table I.

On the twenty-eighth day of fermentation there were present in 100 g. of the decaying straw 26·26 g. of humus containing approximately 18·9 g. of lignin fraction. On the eighty-second day of fermentation the humus of 100 g. of decaying straw had increased to 31·55 g. containing 16·22 g. of lignin fraction. During the whole of this period of intensive humification therefore the lignin fraction had remained practically constant, while the carbohydrate fraction had doubled—from 7·3 g. in the humus of straw after 28 days' fermentation to 15·33 g. in the humus of straw after 82 days.

At the time of the presence of these 15·33 g. of carbohydrate humus in the decaying straw a loss of 20·33 % of hemicelluloses and of 16·08 % of cellulose had been suffered by the original straw. With no information in existence on the efficiency with which carbohydrates are humified under natural conditions it was impossible to say whether the losses of hemicelluloses (pentosans) or those of cellulose were sufficient to account for the formation of these 15·33 g. of carbohydrate humus.

Light can be thrown on this question, however, by examining the position at an earlier stage of the decay, after 28 days of fermentation.

In the sample of straw taken after 28 days there were present in 100 g. of straw 7·36 g. of carbohydrate humus. At this stage the straw had lost 12·33 % of hemicelluloses (pentosans), an amount which conceivably might have been sufficient to yield 7·36 g. of humus. It is unfortunate that evidence cannot be brought forward in support of this. During the same period of fermentation the straw had lost only 3·92 % of cellulose, an amount which even under the most favourable conditions could not have yielded 7·36 g. of humus per 100 g. of straw. It is evident therefore, that, whether responsible for the production of the whole of the carbohydrate humus or not, the hemicelluloses (pentosans) must have taken part in its formation. This conclusion is in agreement with the views of Stoklasa [1898] and of Omelianski [1905] who regard pentosans as particularly important for the increase in the fertility of the soil.

The interesting question of the fate of the carbohydrate fraction of natural humic substances during the process of coal formation unfortunately did not come within the scope of the present enquiry. In a few preliminary experiments it was possible to show that the regenerated humus of three samples of bituminous coal gave chlorine derivatives containing on an average 35·36 % of chlorine. These derivatives, which were of a deeper orange appearance than those of humus derived from recently decayed plant tissues, were entirely soluble in ether. They did not therefore contain typical carbohydrate fractions. The fact that their chlorine content was of the order of that of a mixture of carbohydrate and of lignin humus may perhaps be taken as an indication that the solubility of the whole of the derivatives in ether cannot be regarded as sufficient support for the conclusion that a carbohydrate fraction was absent in these cases.

The regeneration of the humus of the three samples of bituminous coal was carried out by a method recommended by Dr Francis of the Safety in Mines Research Board, Sheffield, in a private communication.

One of the coal samples labelled "Ellistown Main Brieze" was received from the Fuel Research Station, East Greenwich, the two others were samples of ordinary household coal obtained from local coal merchants.

The regenerated humus from the Ellistown sample amounted to 69·44 % of the coal. The corresponding figures for the two other coals were somewhat higher owing to the lower ash content of these coals.

While the above investigation was in progress, a sample of mummified tissues, consisting of a human vertebra with adhering muscular tissues was received by Mr H. J. Bunker of this laboratory from Mr A. Lucas, formerly Director of the Chemical Department of the Egyptian Government, and an opportunity was taken to establish the nature of, and to examine the changes which had occurred in, these tissues.

The material dated from about 200 B.C. and was stated to have become completely humified. On examination its muscular tissues were found to possess an alkali solubility of 82·1 %, while its humus content amounted to 33·8 %.

On chlorinating, the humus took up 22·81 % of chlorine. The chlorine derivative was very similar in appearance to the chlorine derivative of the carbohydrate fraction of vegetable humus. It resembled it also in being almost insoluble in ether.

An ultimate analysis showed the chlorinated humus from mummified muscular tissues to contain 14·16 % of C; 3·62 % of H and 22·81 % of Cl corresponding to a formula of $C_{21}H_{21}O_{13}Cl_4$, the formula of the chlorine component of the carbohydrate fraction of vegetable humus.

SUMMARY.

The early stages of humification of plant tissues (oat-straw) as induced by micro-organisms have been studied. It has been shown that the hemicelluloses (pentosans) of the raw material are at least partly responsible for the appearance of the carbohydrate fraction of the humus.

In three samples of bituminous coal no definite proof could be obtained of the presence of a carbohydrate fraction in the regenerated humus, though the percentage of halogen in the chlorine derivatives of these humus samples was of the order characteristic of a mixture of carbohydrate and lignin humus.

The muscular tissues adhering to a mummified human vertebra dating from 200 B.C. had been converted into a humus with the properties of a typical carbohydrate humus.

The above experiments arose out of work carried out on behalf of the Fuel Research Board of the Department of Scientific and Industrial Research. The authors desire to express their thanks to the Department of Scientific and Industrial Research and to the Admiralty for permission to publish the results obtained.

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CXXIII. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

I. THE SYNTHETIC CULTURE MEDIUM.

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(*Received June 11th, 1927.*)

THIS investigation had as its main object the study of the influence of the ill-defined growth-promoting factor known as bios on the development of unicellular organisms in artificial media. In the early stages it became apparent that no appreciable progress could be achieved until the other essential requirements had been more clearly defined. In particular, this referred to their needs for inorganic salts and the most suitable sources of carbon compounds from which they could derive the necessary energy for their metabolic processes.

It is a curious fact that, in spite of a very large amount of work reported in the literature, the question of the most suitable composition of the artificial medium in which a particular organism can be grown is undecided. This is particularly true of the inorganic composition of the culture fluid, and there seems to be a general impression that the requirements of yeast and bacteria for mineral salts are moderately elastic [Pasteur, 1860; Liebig, 1871; Wildiers, 1901; Windisch, 1902; Pringsheim, 1906; Osborne and Wakeman, 1919; Miller, 1921]. From the experiments reported below it must be concluded that only within a very narrow range of concentration is maximum growth obtained.

EXPERIMENTAL.

Preliminary experiments showed that all the organisms tried would grow on a medium consisting of pure glucose, certain inorganic salts, and water when these were added to clean flasks, sterilised on three successive days, inoculated, and incubated. Consequently it was decided to study in detail the basal requirements of three organisms and find how far (1) the inorganic salt content, and (2) the source of carbon could be varied.

The organisms used were *Sarcina aurantiaca*, *Streptothrix corallinus*, and a white *Streptothrix* as supplied by the National Collection of Type Cultures.

The strains to be used were grown on the surface of broth agar, and carefully plated to make sure that they were pure cultures. Inoculations were always made by suspending some of the dry surface growth in water, using a platinum loop. To avoid acclimatisation effects a fresh culture was used after every four or five subcultures had been made. All water used for making up the media or for the suspensions was prepared from tap water, by distilling, boiling for 3 hours with alkaline permanganate, and redistilling, only glass or silica vessels being used throughout.

The chemicals used were either Kahlbaum's chemicals "Pure for Analysis" or "Analytical Reagents" from the British Drug Houses. The NaCl and $MgSO_4$, $7H_2O$ were further recrystallised. The glucose employed was the "Puriss" variety from the British Drug Houses, but it was found to need four recrystallisations from 80, 75, 70 and 65 % of alcohol before it was rendered free from detectable traces of impurities having a definite stimulating action on growth.

Optimum hydrogen ion concentration. The limiting p_H was found for each organism by inoculating a series of plain broth media tubes and plates of different p_H before attempting to make up a suitable synthetic medium. The approximate limits were found by grading the series by means of colorimetric methods; later a more accurate adjustment was obtained by readings with the glass electrode [Kerridge, 1925]. A series of flasks for each organism was prepared, the contents of the flasks differing from each other by a p_H of 0·02 over the range 4·50 to 10·00. The results were as follows:

Organisms	Lowest limit at which appreciable growth occurred	Maximum growth	Highest limit
	p_H	p_H	p_H
1. *S. aurantiaca*	5·30	7·15	9·43
2. *Streptothrix corallinus*	6·02	7·60	9·35
3. White *Streptothrix*	6·02	7·60	9·35

Later these values were checked when a satisfactory artificial medium had been elaborated and were found to be substantially correct. Meanwhile it was decided to work at a value of $p_H = 7·4$ throughout.

The inorganic salt content. Having established the optimum hydrogen ion concentration for growth, a series of experiments was carried out in which varying quantities of inorganic salts were added to a medium containing 1 % of dextrose.

Table I.

Medium: Glucose 1 %
KH_2PO_4 0·1 %
K_2HPO_4 to $p_H = 7·4$.

	Percentage concentration											
Salt	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	*J*	*K*	*L*
$(NH_4)_2SO_4$	0·1	0·2	0·4	0·1	0·2	0·4	0·1	0·2	0·4	0·1	0·2	0·4
$MgSO_4$, $7H_2O$	0·02	0·02	0·02	0·05	0·05	0·05	0·07	0·07	0·07	0·09	0·09	0·09

(1) A series of flasks was arranged with 12 variations as seen in Table I. Six flasks were prepared for each group in an attempt to eliminate errors in inoculation, etc.

Growth at the end of 7 days was measured by opacity and found to be much better in cultures *E*, *F*, *H*, and *I* than in any of the others. Since in cultures *D* and *G* growth was less good than in *E* and *H* it was concluded that 0·1 % of $(NH_4)_2SO_4$ was not sufficient. It also seemed apparent that the optimum concentration of $MgSO_4, 7H_2O$ lay between 0·05 and 0·09 %.

(2) All the ammonium-magnesium combinations in Table I were duplicated (*a*) with twice, and (*b*) with half the concentration of phosphates

$$(0{\cdot}1\ \%\ KH_2PO_4 + 0{\cdot}016\ \%\ K_2HPO_4),$$

but in neither case was the growth as good as in *E*, *F*, *H* and *I* above. It was therefore concluded that no advance could be made by altering the initial figure for phosphate concentration.

(3) The influence of the addition of varying concentrations of NaCl was next considered. A series of cultures similar to *D*, *E*, *F*, *G*, *H* and *I* was prepared, but containing in addition concentrations of NaCl varying from 0·01 % to 1 %. The cultures containing 0·05 % showed the best growth.

(4) A few preliminary experiments having indicated that traces of $Ca(NO_3)_2$ seemed to be beneficial, it was decided to set up a series containing $Ca(NO_3)_2$ over a range of concentrations from 0·001 % to 1 %. The improvement was very slight in the lower concentrations and growth was definitely retarded with concentrations above 0·075 %. The action seemed independent of concentration below 0·05 %.

(5) Innumerable examples occur in the literature of the last 20 years, some claiming, some disclaiming, the stimulating effect of traces of manganese and iron in the soil or culture medium on the growth of plant tissue. Experiments were designed in which traces of manganese salts, and ferrous and ferric salts were added to the glucose-water-inorganic salt medium described above. The substances used and the results obtained can be seen in Table II.

Table II.

Substance used	Concentration g. in 10 cc.	Increase in growth
MnO_2	1×10^{-4}	Nil
MnO_2	1×10^{-6}	„
$MnSO_4$	1×10^{-4}	„
$MnSO_4$	1×10^{-6}	„
$FeSO_4$	1×10^{-4}	„
$FeSO_4$	1×10^{-6}	„
$FeCl_3$	1×10^{-4}	Slight
$FeCl_3$	1×10^{-6}	„

The slight increase in growth with $FeCl_3$ was probably due to impurities, for after further purification (solution in ether, evaporation of the ether, and volatilisation of the salt) no sign of any action was detectable.

Conclusion from experiments with varied amounts of inorganic salts. From the experiments recorded above it was decided to use the following salt-mixture when studying (1) the effect of variation in the amount of glucose present, and (2) the most suitable source of carbon supply:

$(NH_4)_2SO_4$	0·30 g. in 100 cc. medium
$MgSO_4, 7H_2O$	0·07 ,, ,,
KH_2PO_4	0·10 ,, ,,
K_2HPO_4	0·016 g. (to bring p_H to 7·4)
NaCl	0·05 g. in 100 cc. medium
$Ca(NO_3)_2$	0·04 ,, ,,

Variation in the amount of glucose present. The arbitrary value of 1 % of glucose was used throughout the earlier experiments. It was decided to test this figure, using the salt concentrations tabulated above. Table III shows the results obtained over the range of concentration 0·01–10 %.

Table III.

Concentration glucose (%)	Growth
0·01	↑ Gradually diminishing
0·02	
0·03	
0·04	
0·06	No appreciable variation
0·08	
0·10	
0·20	
0·30	
0·40	
0·50	
0·60	
0·70	
0·80	
0·90	
1·00	
2·00	
3·00	↓ Gradually diminishing
4·00	
5·00	
6·00	
7·00	
8·00	
9·00	
10·00	

0·5 % of glucose was used for all later experiments.

Influence of source of carbon. Although the medium described above gave good results, it was thought of interest, in view of the number of publications on this subject, to study the value of sources of carbon other than glucose. The growth with glucose as the source of carbon is taken as standard and recorded by two crosses, *e.g.* ++.

Three crosses = increased amt. growth.

One cross = decreased amt. growth.

Table IV.

Source of carbon (0·5 % solution in salt-water medium)	Growth	Source of carbon (0·5 % solution in salt-water medium)	Growth
Glycerol ...	+ + +	Sodium pyruvate	+ +
Mannitol ...	+ + +	Fructose	+
Glucose ...	+ +	Rhamnose ...	+
Arabinose ...	+ +	Sodium acetate ...	+
Glycogen ...	+ +	„ propionate	+
Calcium lactate	+ +	„ butyrate	Trace
Sodium lactate...	+ +	„ oxalate ...	Trace
„ citrate...	+ +	„ succinate	0
		„ tartrate ...	0

The growth on glycerol and mannitol calls for comment. Although the suspension was more opaque, the rate of reproduction did not seem to be increased, but the growth was more filamentous, in fact almost vegetative. Whereas the segmentation of the *Streptothrix* usually begins about the third day, it could be delayed to the seventh or eighth day with mannitol or glycerol as the source of carbon. The effect of citrate is surprising, but the presence of mycelial threads in the culture fluid points to an effect similar possibly to that of glycerol or mannitol.

Amino-acids as source of carbon or nitrogen. The following experiments were designed to test the amino-acids (1) as a source of carbon, and (2) as a source of nitrogen for bacteria. Column 1 in Table V shows results obtained when the glucose was replaced by a 1 % solution of the various amino-acids tabulated. Column 2 gives results when the ammonium sulphate was replaced by the amino-acids, the glucose being added as usual.

The growth obtained with glucose (0·5 %) and ammonium sulphate (0·3 %) present was again used as standard.

Table V.

	Growth	
Amino-acid (0·5 % solution)	Column 1 Amino-acid as source of carbon	Column 2 Amino-acid as source of nitrogen
Tryptophan ...	–	–
Tyrosine	–	–
Alanine	+ +	Trace
Phenylalanine ...	–	„
Histidine	–	„
Cystine*	+	„
Glycine	+ +	„
Asparagine ...	+ +	„
Glutaminic acid ...	–	„
Tyrosine + alanine	+ +	„
Tyrosine + histidine	–	–
Alanine + histidine	+ +	+
Histidine + glycine	+ +	Trace

* The low solubility of cystine enabled only a 0·1 % solution to be used. The flasks were tested at the end of the experiment and cystine found to be still available.

In column 1, *i.e.* in the absence of glucose, growth was obtained with alanine, glycine, asparagine, and to a less degree with cystine.

In column 2, *i.e.* in the absence of ammonium salts, the growth was erratic and was certainly never of the same order as with a purely inorganic source of nitrogen.

DISCUSSION.

The experiments recorded in this paper show that it is comparatively easy to obtain growth of the organisms studied on a synthetic medium, but whether the metabolism and rate of multiplication of the organisms are normal under these conditions remains to be proved. In all cases it was found possible to obtain a marked improvement of the rate of growth by adding minute quantities of broth or of any other bios-containing substance. Experiments on the effect of bacterial accessory factors will be discussed in a later paper.

The absolute qualitative requirements of the organisms studied seem to be fairly simple. It is indeed true that the formula worked out for the inorganic requirements of the culture medium differs only slightly from that of Stephenson and Whetham [1924], but what the author wishes to emphasise is the necessity for keeping within narrow limits the range of concentration of any one ingredient. The presence of Cl ions is essential and a trace of $Ca(NO_3)_2$ seems to encourage growth.

It is impossible to exaggerate the importance of the progressive purification of the glucose. The filtrate after a first recrystallisation can be shown to have a distinct stimulating effect upon the growth: the filtrate after the fourth recrystallisation has none. A similar action was pointed out by Funk and Dubin [1921] in their experiments on the stimulating effect of "vitamin B" on the growth of yeast and bacteria. They state that treatment of the sugar with an absorbent eliminates the effect. This has recently been confirmed, and it was found that after treating the glucose with charcoal only two recrystallisations were necessary.

The addition of amino-acids to the synthetic glucose-inorganic salt medium, being an addition of heavier and more complex molecules, naturally complicates the problem. In this connection the work of Aubel [1920] is interesting but his view has not been confirmed. He studied cultures in which asparagine was the only source of carbon and of nitrogen, and compared them with cultures containing also glycerol, glucose and fructose. More ammonia was used and more growth was obtained by these additions. Glycerol gave a ninefold increase; this he believed to be due to the formation of pyruvic acid, which, given directly, had nearly twice the augmenting power of glycerol. His conclusion, therefore, was that carbon as —CO is most effective, then comes —CHO and lastly —CH_2OH. On the other hand, the conclusion to be drawn from the experimental section of this paper is that pyruvate is not more efficient in promoting growth than glucose, and is obviously inferior to glycerol.

The possibility that the controlling factor in the variation of growth is a purely physical one, such as osmotic pressure, surface tension, etc., must be considered. That the lowering of surface tension within certain limits by an addition of fatty compounds may alter the type, but not the amount, of growth is shown in the following paper [Reader, 1927].

SUMMARY.

1. Experiments have been carried out to find the concentration of salts in a synthetic medium necessary to give maximum growth of certain micro-organisms. The organisms used were *Streptothrix corallinus*, a white *Streptothrix*, and *Sarcina aurantiaca* and a definite formula was decided upon.

2. Traces of manganese or iron did not increase the rate or amount of growth.

3. The following substances may replace glucose as source of carbon without loss of efficiency in the medium: glycerol, mannitol, arabinose, lactate, citrate, pyruvate and glycogen.

4. Amino-acids, as such, do not supply an improved source of nitrogen or carbon to the medium. Glycine, asparagine, alanine, and possibly cystine may supply carbon, but ammonium salts are a more efficient source of nitrogen.

I take this opportunity of expressing my thanks to Prof. J. C. Drummond for his helpful advice and criticism throughout this work, and to Mrs P. T. Kerridge for help with the determinations of hydrogen ion concentrations.

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CXXIV. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

II. THE EFFECT OF CHANGES OF SURFACE TENSION ON GROWTH.

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DURING the growth of cultures of *Streptothrix corallinus* upon the basal medium [Reader, 1927] with addition of broth or antineuritic concentrates (torulin), it was noticed that the organisms were distributed sometimes at the surface and sometimes throughout the volume of the fluid. This rather haphazard phenomenon suggested the effect of a surface tension factor, and experiments were accordingly devised to determine whether surface tension had an effect upon (1) the amount of growth, and (2) distribution or type of growth.

Previous work upon the subject of surface tension has been summarised by Frobisher [1926]. He draws attention to the significance of surface tension, not only in modifying the growth and morphology of bacteria, but perhaps also in influencing their cell-division, motility, and retention of Gram's stain. He found that differences in ability to grow at low surface tensions provided a simple method for distinguishing species of bacteria. Thus, by comparing the growth of organisms at low surface tensions, he succeeded in distinguishing *B. aerogenes* from *B. typhosus* and *B. morgani*; *B. diphtheriae* from *B. xerosis* or *B. hofmanni*, certain resistant types of streptococci from the more sensitive types. The raising (by the addition of charcoal) of the surface tension of Huntoon's hormone medium for pneumococci was, he claimed, responsible for an improvement of its growth-promoting properties.

Before the publication of Frobisher's results the only direct studies of the effect of surface tension on bacterial growth had been those of Larson and his colleagues. Larson, Cantwell and Hartzell [1919] claimed that the growth of pneumococci and streptococci was, in general, inhibited by surface tensions below about 50 dynes. They had also observed that the growth of *B. anthracis* was checked by a surface tension below 46 dynes, and that the sporulation of *B. subtilis* was similarly affected. It is, however, unfortunate that they did not substantiate their report by a detailed record of experiments. Larson and Larson [1922] studied the rôle of surface tension in pellicle formation. Larson and

Montauk [1923], cultivating tubercle bacilli in a medium containing soap and glycerol, noticed that the organisms grew indeed in this medium, producing a diffuse cloudiness, but appeared to have lost all pathogenicity, so much so that their injection into guinea-pigs was followed by nothing more than local temporary nodules. Tubercle bacilli in sputum seemed likewise to lose pathogenicity when treated with sodium ricinoleate. These results lend plausibility to the suggestion that low surface tension may have been a determining factor in the experiments of Calmette and his colleagues [1921].

Thus previous work shows that there is some evidence of a correlation between the amount of growth and the surface tension of the medium, but there appears to be no reference to any relation to the distribution of the organism throughout the medium

EXPERIMENTAL.

Surface tension was determined by a drop volume method using a glass tip of approximately 3·8 mm. external diameter. The technique for cultivation and estimation of the growth of the organism, *Streptothrix corallinus* [Reader, 1926, 1, 2], was the same as described in the previous paper [Reader, 1927]. The broth employed was made from fresh ox-heart, trypsinised according to the method of Douglas. The antineuritic concentrates used were provided by Kinnersley and Peters [1925]. Small conical flasks (60 cc.) were used throughout, and the final volume of the medium was always made up to exactly 20 cc. in an attempt to keep the surface area constant.

Two types of measurement of surface tension were employed: (*a*) the surface tension between the medium and air, and (*b*) the interfacial tension between the medium and benzene. The latter measurement was undertaken under the belief that this figure might represent more nearly the change at the medium-cell interface. In view of the experiments of Hartridge and Peters [1922] the p_H was kept constant throughout. From Table I it will be seen that the percentage change is much greater in the interfacial surface than in the air surface for any one set of dilutions of medium. In all later experiments it was decided to use the interfacial medium/benzene figure, which seemed to be a more significant value and as it gave larger differences therefore decreased the percentage experimental error.

Table I.

	Pure medium	+ 1/200 broth	+ 1/20 broth
Volume of drop into air (cc.)	0·057	0·056	0·049
Volume of drop into benzene (cc.)	0·202	0·163	0·116

The figures in Table II are measurements of interfacial tension immediately *before* inoculating the flasks with the organism and incubating for 2 days at 31°. It was found that the interfacial tension against benzene did not change even during a prolonged growth period of 4 weeks, suggesting that the factor lowering interfacial tension is not utilised in growth. α, β and γ represent in descending order the amount of growth in 48 hours gauged by opacity, standard flasks of a definite extract in varying concentrations being used for comparison. In these, growth was arrested after 48 hours by addition of formalin.

Table II.

Temperature =16·8°
p_H =7·4
Time of 1 drop=40 seconds

Dilution of broth in salt-sugar medium	Volume of drop into benzene cc.	Growth in 48 hours at 31°	
1 in 0	0·057	α	Film
1 ,, 1	0·058	α	,,
1 ,, 10	0·097	α	,,
1 ,, 20	0·116	α	,,
1 ,, 40	0·127	α	,,
1 ,, 60	0·134	β	,,
1 ,, 80	0·139	β	,,
1 ,, 100	0·144	γ	,,
1 ,, 200	0·163	decreasing ↓	,,
1 ,, 300	0·175		,,
1 ,, 400	0·186		,,
1 ,, 500	0·199		,,
1 ,, 1000	0·202		Deposit
0 ,, 1	0·202		,,

From Table II it may be observed that a gradual increase in the percentage of broth added to the synthetic medium increased growth up to a certain definite strength (1 in 40) when it was already equal to that obtained in a full broth culture; but whereas the addition of broth lowers the surface tension even when the dilution has reached only one part in 500, this is a figure widely divergent from that for maximum growth (1 in 40) recorded above. Further, when the interfacial tension is lowered even 1·5 % below its initial value (0·199 cc.) the character of growth changes from deposit to film.

Consequently it seemed probable that the amount of growth and the type of growth were not dependent upon the same factor. The film-growth seemed to be due to the lower surface tension of the medium rather than to the desire of the organism for more atmospheric oxygen, nitrogen, or other substance as is so often suggested.

Fig. 1 shows the type of curve obtained with descending dilutions of broth in a synthetic medium. Since the density of the solutions is not appreciably altered until a high concentration is reached, the surface tension can be considered for this experiment as directly proportional to the volume of drop except possibly for a slight error in the first point of the curve.

That the amount and the type of growth are in no way correlative is more definitely shown in Table III. The results there cited are typical examples of a series of fifty such experiments. The letters *A*, *B*, *C*, etc. represent various torulin concentrates and were used merely as sources of bacterial accessory factors. The figures in the second column are relative values only, 0·202 cc. being the value for pure inorganic-glucose medium. It will be noticed that whenever the surface tension is low a surface film-growth is obtained; but the actual records of growth (α, β, γ, etc.) by no means correspond with the surface tension, or with the presence of films.

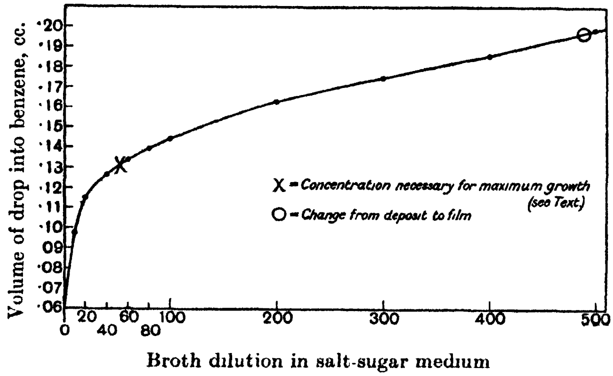

Fig. 1.

Table III.

Medium	Volume of drop (cc.)	Growth	Type of growth
A	0·105	α	Dense surface film
B	0·202	α	Rich deposit
C	0·151	$\alpha -$	Dense surface film
D	0·202	$\alpha -$	Rich deposit
E	0·127	β	Film
F	0·202	β	Deposit
G	0·184	γ	Film
H	0·199	γ	Deposit

Discussion.

The above experiments show that, when ordinary media are used, the surface tension of the fluid (within the limits recorded) does not affect the amount of growth. These conclusions may seem to be at variance with the work of Frobisher, of Larson, Cantwell and Hartzell [1919], and of Wolf [1923], but it must be remembered that their experiments involved the addition of substances such as soap and alcohol, and are, therefore, beyond the scope of this paper.

As there is no correlation between change in surface tension and amount of growth, it must be concluded that the growth-promoting effect of the medium used was not due to any surface action.

Summary and conclusions.

1. As the amount of growth of *Streptothrix corallinus* in synthetic medium containing additions of broth or antineuritic concentrates (torulin) is not related to the change of surface tension in air or interfacial tension against

benzene, the growth-promoting activity is not due to lowering of tension of the medium.

2. The type of growth (film or deposit) is correlated with change in interfacial tension of the medium against benzene.

I am indebted to Mr Kinnersley and Professor Peters for the torulin concentrates used throughout this work. I also wish to thank Professor Peters for his interest and helpful criticism.

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CXXV. EXPERIMENTS ON NUTRITION. VI.

BALANCE OF FOOD BY VITAMIN B.

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(*Received June 14th, 1927.*)

Our previous experiments [1922, 1923] on the rearing of chicks have indicated that the quantity of vitamin B required by the birds must bear a relation to the quantity of food consumed. These observations were thus similar to those described by Braddon and Cooper [1914]. Braddon [1907] had previously pointed out that in the epidemics of beriberi in the East the well-nourished succumbed first and were more liable to the disease than the underfed. Men with their greater energy output and consuming more rice than women were more subject to the disease. If men and women were kept upon the same fixed ration, as in institutions, both were equally susceptible. If they were supplied with equal quantities of other foodstuffs, an increase in the amount of white rice was followed by more cases of beriberi. The time of the appearance of the typical paralysis has been seen also to vary with the climatic conditions. Cold and wet weather, as well as exercise, hastened the onset, whilst warmth and rest delayed the appearance of the typical symptoms.

A connection between the actual quantity of food eaten and the time of onset of paralysis in birds receiving no vitamin B was first pointed out by Maurer [1907]. Cooper [1913] also observed that the disease appeared sooner in pigeons on larger amounts of white rice than in those on smaller amounts. Funk [1914] showed clearly that the onset of polyneuritis in pigeons occurred much sooner on 20 g. of rice than on 5 g., and also that on a constant amount of food polyneuritis appeared most quickly with starch or sugar, more slowly with caseinogen and lastly with fat.

The metabolic connection between carbohydrate and vitamin B was investigated in some detail by Braddon and Cooper [1914]. From their experiments with fowls it appeared that the greater the amount of carbohydrate, the greater was the amount of vitamin B required. Their work was never completed. No other direct experiments upon these lines had been made at the time that our experiments were started. The question of a quantitative relation between food intake and vitamin B has seemed to us of so much importance in nutrition that our work during the past 3 or 4 years has been to test it, using each kind of foodstuff, protein, fat and carbohydrate.

In the meantime several other papers have appeared which show that vitamin B is concerned in the metabolism of food. Karr [1920] and Cowgill [1922] pointed out a relation between quantity of vitamin B and appetite. Osborne and Mendel [1922] found a connection between body weight and quantity of vitamin B. This connection has been elaborated by Cowgill [1922], Cowgill, Deuel and Smith [1925], Cowgill, Smith and Beard [1925] and by Beard [1925]. Randoin and Simonnet [1924] found a direct relation between carbohydrate consumption and amount of vitamin B. Hartwell [1924, 1925] and Reader and Drummond [1926] found a relation between quantity of protein in the food and amount of vitamin B. Our results point to the need of balancing all classes of foodstuffs by vitamin B, *i.e.* the supply of vitamin B must be in proportion to the calorie value of the food. Various pathological symptoms are seen which appear to originate from too little, or a shortage, of vitamin B in the food over a long period.

EXPERIMENTAL.

The rearing of chickens from day-old to maturity has been taken as the test to ascertain the balance of the three foodstuffs, protein, fat and carbohydrate by vitamin B. The rearing of ducks has been employed in additional experiments on balance of protein. In the case of balance of carbohydrate, pigeons and rats have also been used.

The housing of the chickens has been the same as described previously [1923]. Day-old chicks and ducks have been started in a foster mother and transferred, according to the weather, in 3 to 5 weeks, to a small house and later to a larger house, all the houses being provided with small runs. The houses have been on the roof of the building and exposed to the weather. Light Sussex chicks have been available for most of the experiments. Leghorns were used in one experiment and in another there was a variety of breeds. The pigeons were of the ordinary homer variety and were kept in pairs in cages, measuring approximately 4′ × 2′ × 2′. The cages were kept side by side on the roof of the building and were fitted with a convenient perch. The rats have been kept in wire cages, measuring approximately 2′ × 1′ × 1′, generally in pairs and housed in a warm room of the laboratory.

The birds and rats have always been fed *ad lib.* They have been provided with food in a tray, and as soon as it was finished, it was replenished. The food consumption could thus be recorded. All animals have been weighed weekly.

I. THE BALANCE OF PROTEIN BY VITAMIN B.

In our previous experiments [1923] it was found that chicks, groups VIII and IX, could be successfully reared on a diet composed of:

White rice	Marmite	Fishmeal	Cod-liver oil	
90	9	5	1	parts.

This diet presumably contained just enough vitamin B in the marmite to balance the rice and the small quantities of fishmeal and cod-liver oil. It has formed the basis of these experiments, which were devised on the following supposition.

Without any change in the above diet except raising the fishmeal to 30 parts, failure to rear chicks would indicate insufficient vitamin B, or some harmful effect of high protein in the food, and that the extra protein needed balancing. Then, by keeping the fishmeal constant at 30 parts and increasing the quantity of marmite, the capability of rearing chicks would show that the extra protein needed balancing with vitamin B and that the high protein was not in itself injurious.

First series of experiments.

Two groups of chicks, XII 5 and XII 30 were started together, the former as a control with 5, the latter with 30 parts of fishmeal. To the diet of group XII 5 was added the ash obtained by burning 25 parts of fishmeal, to represent the extra mineral matter supplied with the 30 parts of fishmeal to group XII 30.

Shortly afterwards, as a few more chicks became available, two more groups, XIII and XIII A, were started, both on low protein but with the proportions divisible into third quantities in order to be able to make up smaller amounts of food. These acted as a further control.

The rearing of group XII 5 was not so good as previously with groups VIII and IX. Groups XIII and XIII A reached maturity. The birds of group XII 30, except two, were not reared and these two were abnormal. More details are given below.

Further groups, XIV, XV, XVI, were then started in succession with increasing quantities of marmite. The results were worse with group XVI than with group XV. It was then suspected that the marmite was not so good as that previously used. Another control group, without and with ash from 25 parts of fishmeal, was then again tried. The results with these groups, XVII and XVII A, confirmed our suspicion that there had been some change in the marmite. Several other samples of yeast extract have in the course of these experiments been tested on pigeons and have been found to vary considerably (see later).

The series of experiments and the general results are best seen and compared by reference to Table I.

Table I.

Group	White rice	Marmite	Cod-liver oil	Fishmeal	No. of birds	Losses
XII 5	90	9	1	5	8	4
XII 30	90	9	1	30	8	6
XIII	90	9	1·5	6	3	0
XIII A	90	9·9	1·5	9	2	1
XIV	90	12	1	30	10	4
XV	90	14	1	30	12	1
XVI	90	16	1	30	10	6
XVII	90	10	1	5	9	5
XVII A	90	10	1	5	11	5

Composition of diets %.

Group	Water	Salt	Other ash	Fibre	Protein	Fat	Carbohydrate	Marmite	Calories per 100 g.	Ratio $\frac{\text{marmite}}{\text{calories}}$
XII 5	12·9	1·4	6·1	0·3	11·2	1·5	70·3	8·6	348	1 : 40
XII 30	13·2	1·7	5·7	0·2	20·8	1·8	56·9	6·9	335	1 : 49
XIII	12·9	1·4	2·7	0·3	11·6	2·0	69·3	8·4	350	1 : 42
XIII A	12·9	1·5	6·1	0·3	13·1	2·0	66·9	8·9	347	1 : 39
XIV	13·5	2·0	5·9	0·2	21·0	1·8	55·7	9·0	331	1 : 37
XV	13·7	2·1	6·0	0·2	21·3	1·8	55·0	10·4	330	1 : 32
XVI	13·9	2·3	6·1	0·2	21·4	1·8	54·4	11·7	328	1 : 28
XVII	12·9	1·5	2·6	0·3	11·5	1·5	69·7	9·4	347	1 : 37
XVII A	12·4	2·0	5·9	0·3	11·1	1·5	67·2	9·1	335	1 : 37

Although more birds were raised on the higher amounts of marmite in the food, the result in respect of balance of protein by vitamin B was not satisfactory and conclusive. The chickens which reached maturity were not normal in any of the high protein groups. They had bow legs, or were knock-kneed, and in some cases there were signs of leg weakness.

The most interesting and important details concerning these groups of birds were observed on *post mortem* examination. The details of the losses and *post mortem* examinations are given below.

Group XII 5. This experiment lasted for 27 weeks, from 15. viii. 1923 to 20. ii. 1924. The four losses occurred in the 4th, 5th, 7th and 18th weeks. A perforated gut was found in the first. In the second, the abdomen was full of liquid, the heart was enlarged, the gizzard small and gut empty. The third bird, before death, showed neck twist, which got better and then worse. It was chloroformed in the 27th week. Its caecum was very swollen. The fourth bird was pale and had drooping wings before death. The intestine was full of dark food material, the gizzard had thin walls and the kidneys seemed large. An extra bird introduced into this group at the age of 6 weeks showed leg weakness and bent legs in the 22nd week. It was chloroformed; its gizzard was thin-walled, intestines leathery and appendices swollen.

Slight leg weakness was noticeable in the 19th week and the group was kept longer, but distinct signs were not noticed in all the birds.

Group XII 30. This experiment was for 21 weeks, from 15. viii. 1923 to 9. i. 1924. The first loss in the 2nd week showed much liquid in the body cavity. The gut seemed normal. The second in the 4th week had an empty intestine: the bones seemed soft. The third in the 5th week had distinct leg weakness. The leg joints were large. The gut was almost empty. The fourth in the 15th week was very wasted. The knee joints were swollen and legs twisted. There was very little muscle on the gizzard. The fifth and sixth died in the 20th week. There were hard lumps in the caeca with stoppage. The gizzards had thin walls. The two surviving birds were kept to the 26th week. No gut symptoms were noticed at autopsy. The ovaries were small. The birds were abnormal in appearance.

Group XIII. The experiment was for 25 weeks, from 11. ix. 1923 to 20. ii. 1924. One bird showed neck twist in the 11th week, but got better. There seemed signs of leg weakness in the 16th week, but it did not get worse. This group with the two remaining hens from group XII 5 were kept altogether for 12 months without any symptoms. The marmite was raised to 10 parts for the latter period.

Group XIII A consisted of only two chicks, one of which was very small and feeble at the start and should not be included. The surviving chick at the 8th week was put in group XIII.

Group XIV. This experiment was continued only for 10 weeks, from 6. ii. 1924 to 16. iv. 1924. Two birds showed badly twisted legs in the 4th week and were chloroformed. They had pale combs and swollen joints. The caeca contained masses of food which caused stoppage. Two further birds showed crooked legs in the 5th and 6th weeks and were chloroformed. The second had water in the body cavity; its intestines and appendices were swollen. All the birds at the 8th week were very weak on their legs. The marmite was raised to 16 parts, but they did not get better and were chloroformed at the 10th week.

Group XV. This experiment lasted for 9 weeks, from 26. iii. 1924 to 26. v. 1924. The loss in this group was in the 2nd week. The bird had a crooked leg and was chloroformed. It had an unabsorbed yolk sac. The other birds had bow legs, or were knock-kneed and weak on their legs. On this account the experiment was not continued longer.

Group XVI. These were Leghorns. The experiment was for 7 weeks, from 22. iv. 1924 to 18. vi. 1924. This was an unsatisfactory group as seven birds died in the 1st week from unabsorbed yolk sacs, which pointed to weakness in the other birds. One bird developed a crooked leg in the 5th week and was chloroformed. Another died in the 7th week. Its abdomen was full of water, but the intestines seemed normal; its heart was normal. All the birds were very ill and the experiment was discontinued.

Group XVII. This experiment lasted for 20 weeks, from 22. iv. 1924 to 10. ix. 1924. The first two losses occurred in the 3rd week. Both appeared to have stoppage of the gut. The third loss was in the 4th week. Both appendices of the bird were clogged with rice. At the 6th week the birds remaining from group XVII A were put in with this group. Two deaths occurred in the 19th week; no special symptoms were noticed. Another loss was in the 21st week. This bird had an enlarged heart with clot in the pericardium; the connective tissue in the abdomen was leathery.

Group XVII A was fed for 6 weeks, from 22. iv. 1924 to 28. v. 1924. The first loss was in the 2nd week. The liver was pale and the gall bladder large. The second and third losses were in the 3rd week. One showed a stoppage of the intestine and the other a distended gut with pale liver and gall bladder. The fourth and fifth died in the 4th week. In one the yolk sac was not completely absorbed and there was stoppage near this point. In the other, the gut was full of hard faecal matter, the liver and gall bladder were pale. The surviving birds were now put in with group XVII.

The general results of these experiments were not conclusive. On the whole the birds seemed to thrive better with the extra marmite in the food. The birds with the high protein diet were always abnormal. It was difficult to account for the abnormalities. Several possibilities could be suggested. They may have been due to the high salt content of the diet, derived from the marmite and from the fishmeal, or to the high amount of the protein, or possibly to too little cod-liver oil, or to a combination of several conditions. The extra mineral matter introduced with the higher amounts of fishmeal was of no value in preventing leg weakness and this does not support the claim that lack of mineral matter is the cause of deficiency diseases. In former experiments a diet had been fed to chicks with an equally high protein content, and no ill-results were observed. It was therefore difficult to attribute the errors to high protein. The amount of cod-liver oil though about 0·7 % of the diet would also appear adequate.

Experiments with ducks.

Some trials of a similar kind were made about the same time with groups of ducklings. As it was previously found that ducks required more marmite than chicks, the quantity of marmite was placed at a higher figure at the start. The diets and general results are given in Table II.

Table II.

Group	White rice	Marmite	Cod-liver oil	Fishmeal	No. of birds	Losses
D, III	90	12	1	30	12	4
D, IV	90	16	1	30	4	0
D, V	90	20	1	30	11	6
D, VI	90	40	1	30	7	0

Ducks III were not well and were shaky on their legs in the 2nd week. On this account, the marmite was slowly raised (every 2 weeks) to 20 parts. It seemed that it was only by these means that the ducks could be reared.

The first loss showed a pale fatty heart; the caecum had stoppage and the appendices were blown. There was stoppage of the gut with the cloaca distended with gas in the second loss. No cause could be found for the third death. The gut was normal in the fourth except that one appendix was full. The interior was pale.

Ducks IV were very shaky on their legs and were combined with ducks V at the 10th week.

Ducks V. All but one of the losses in this group occurred in the 3rd week. The only symptoms seemed to be swollen and pale kidneys. There was a loss in the 10th week apparently from tuberculosis.

As a final trial ducks VI were fed with a very high amount of marmite. They were all reared to the 10th week and were quite normal.

Altogether there was more evidence for balance of protein by vitamin B in these experiments than in the corresponding ones with chickens.

Second series of experiments with chickens.

Owing to the apparent deterioration of the marmite, this series of experiments was made with dried yeast as source of vitamin B. Its use would also get rid of the possibility of the high salt content of the previous diets. It had been found previously [1923] that dried yeast contained about half the amount of vitamin B present in the former marmite, so that 20 parts were taken as the equivalent of 10 parts of marmite as the minimum quantity to balance the basal diet. This series was carried out in the reverse order starting with high yeast and diminishing as shown in Table III.

Table III.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XIX	90	32	1	30	11	0
XX	90	28	1	30	11	0
XXI	90	24	1	30	10	0
XXII	90	20	1	30	10	2

Composition of diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Yeast	Ratio $\frac{\text{yeast}}{\text{calories}}$
XIX	10·1	5·8	0·2	24·8	1·7	56·8	350	21	1 : 17
XX	10·3	5·7	0·2	24·3	1·7	57·1	349	19	1 : 18
XXI	10·5	5·6	0·2	23·7	1·8	57·5	350	17	1 : 21
XXII	10·8	5·6	0·2	23·2	1·7	57·9	348	14	1 : 25

All these experiments were made in succession for a duration of 10 weeks during the time 27. vi. 1924 to 16. ix. 1924.

Though no losses occurred in the first three groups, all the birds at the age of 10 weeks had either bow legs or were knock-kneed, but otherwise they were fairly normal.

In group XXII, in one of the birds lost, there was a twist in the intestine, probably an abnormality at the start as the bird died in the 2nd week. In the other bird the gut seemed normal, but the kidneys were large and uric acid was found by testing in the ureters and cloaca.

The results again were not clear. The high protein seemed to have a deleterious effect. The birds seemed to be distinctly better on the higher amounts of dried yeast.

Third series of experiments.

This series was made to ascertain the effect of increasing quantities of protein in the diet so as to find out whether the high protein was producing the bow legs and other symptoms. The amount of dried yeast was kept constant (Table IV).

Table IV.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XXIII	90	20	1	5	9	0
XXV	90	20	1	10	9	2
XXVI	90	20	1	15	7	0
XXVII	90	20	1	20	9	4
XXVIII	90	20	1	25	13	2
XXIX	90	20	1	30	8	2
XXX	110	20	1	5	12	4

Composition of diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Yeast	Ratio $\frac{\text{yeast}}{\text{calories}}$
XXIII	9·9	2·8	0·2	15·1	1·5	66·9	350	17·2	1 : 20
XXV	10·1	3·4	0·2	17·0	1·5	64·1	346	16·5	1 : 21
XXVI	10·3	4·0	0·2	18·7	1·6	61·6	344	15·9	1 : 22
XXVII	10·5	4·6	0·2	20·3	1·7	59·2	342	15·3	1 : 22
XXVIII	10·6	5·1	0·2	21·8	1·7	57·1	339	14·7	1 : 23
XXIX	10·8	5·6	0·2	23·2	1·8	55·1	338	14·2	1 : 24
XXX	10·1	2·4	0·3	13·9	1·3	69·0	352	14·7	1 : 24

The last group acted as a control with extra white rice equal to 30 parts of fishmeal. All the groups were kept for 20 to 23 weeks, from 21. vii. 1924 to 4. v. 1925.

Group XXIII. All the birds were reared without any signs of illness. They were actually continued for another 10 weeks with one cock bird and laid eggs. They had a tendency to eat the eggs. On this account the cod-liver oil was raised to 2 parts for a further 7 weeks and then to 3 parts for a period of 5 weeks. This increase lessened the desire to eat eggs.

Group XXV. The *post mortem* examination of the two birds which died in this group did not reveal any particular symptoms. Almost the same diet was used again in group XXXIII and the birds were reared satisfactorily.

Group XXVI. This group was reared quite satisfactorily.

Group XXVII. This group turned out to be composed only of cock birds. The first loss was in the 4th week. The gut contained compact food masses and the appendices were swollen. The other three birds had crooked legs at the end of the period of experiment and were chloroformed. All three were found to have swollen appendices with dark food residues in them; the livers were large and fatty. The other birds seemed normal.

Group XXVIII. This group looked ill in the 7th week and the birds were shaky on their legs. Several showed only small increases in weight at the 13th week. The first loss was in the 19th week. The bird had enlarged kidneys, stopped gut and very swollen appendices. The other died in the 23rd week and had also stopped gut and swollen appendices. The hen birds of this group showed bow legs or legs with slight twist.

Group XXIX. Like group XXVIII the birds showed signs of illness in the 7th week. All the birds were shaky on their legs and the legs of many were crooked. The first loss was in the 19th week; examination showed a flabby gizzard and one swollen appendix. The other bird died in the 20th week. Its appendices were swollen, the liver was fatty and there was much fat upon the heart.

Group XXX. This group, a control group to XXIX, showed its first loss in the 4th week. There was some stoppage of the large intestine and the appendices were full of black food residues. The second loss was in the 5th week. There was undigested rice in the large intestine and the appendices were large. The third bird died in the 6th week. The large intestine was full of food residues, but the appendices were empty. The fourth bird showed bent legs in the 9th week; it did not get better and was killed in the 17th week. Its abdomen was found full of water and gelatinous matter.

All birds of the group were more or less shaky on their legs at the end of the experiment.

It appears from these experiments that high protein affects the birds and may be the cause of the bow legs or knock knees. These symptoms do not appear until the fishmeal reaches 20 parts, or about 20 % of the food. A clear demonstration of the need of balancing the protein by vitamin B does not seem possible without producing ill-effects from too much protein. Excluding the leg abnormalities, the *post mortem* findings were very similar with the smaller amounts of vitamin B, and were generally concerned with the intestines and appendices and sometimes the heart.

Fourth series of experiments.

During the course of the previous experiments others were made with small amounts of cod-liver oil and it was being noticed that 0·4 to 0·6 % was too little and later that 0·5 to 0·7 % of cod-liver oil was enough to rear birds without any signs of illness. In the experiments with low fishmeal, the amount of cod-liver oil was 0·9 %. This amount became reduced to 0·8 and 0·7 % as the fishmeal increased in quantity. It was possible that too little cod-liver oil was the cause of the leg abnormalities. This series was tried with 2 % of cod-liver oil so as to avoid ill-effects from too little. The diets used in these groups were made to contain 2 % of cod-liver oil and the whole diet was made out to a total of 100 parts. The diets are shown in Table V.

Table V.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XXXIII	74	16	2	8	10	1
XXXV	78	12	2	8	10	2
XXXIV	56	12	2	30	10	2
XXXVII	52	16	2	30	12	3

Composition of the diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbohydrate	Calories per 100 g.	Ratio ——— calories
XXXIII	10·0	3·3	0·2	16·6	2·7	66·9	368	1 : 23
XXXV	10·4	3·0	0·2	15·1	2·7	68·4	367	1 : 31
XXXIV	11·1	6·8	0·2	27·0	3·3	50·6	349	1 : 29
XXXVII	10·7	7·3	0·2	28·5	3·3	49·1	349	1 : 22

The diet of group XXXIII was the same as that of group XXV except for the cod-liver oil. With group XXXV this group acted as control to the two groups with high protein.

Group XXXIII. The one loss in this group could be accounted for by an accident. All the birds were perfectly fit and this one showed one morning peculiar symptoms of turning somersaults. The *post mortem* examination showed normality in all the organs. The bird was probably pecked by another or had some injury to its head.

Group XXXV. The losses were in the 5th and 6th weeks. The first was shaky on its legs and showed slight leg weakness. At autopsy, decomposing food was found in the large intestine extending into the small intestine. The appendices were full and swollen. The second bird had excessive amounts of fat on the abdominal organs. The intestines were blocked and the heart was very large. The other birds of the group were quite normal.

Group XXXIV. All the birds in the group showed leg weakness and looked very ill. The experiment was consequently stopped in the 15th week.

The two losses were in the 5th and 7th weeks. Both birds were chloroformed on account of their leg weakness. The first had decomposing food in the large intestine and swollen appendices; the gizzard was flabby and full of water. There was gas in the appendices of the second bird.

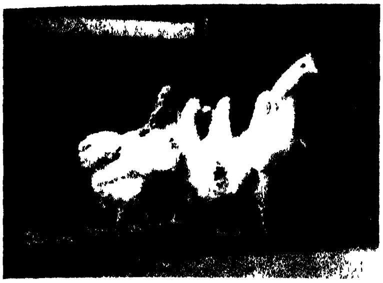
Group XXXVII. The experiment was stopped at the 16th week as all the birds showed bow legs. The first loss was in the 3rd week. The intestines and appendices were full of dark food residues. The second died in the 13th week. There was ballooning of the gut near the gizzard. In both birds the hearts seemed small.

Group XXXVII. These birds were generally in better condition than those of group XXXIV. They were able to walk whereas those of group XXXIV could not do so. It would have been quite possible to continue group XXXVII, but no further information was expected.

As the cod-liver oil was raised in this series, the abnormalities in the legs thus appeared due to high protein in the diet. The birds were distinctly better with the higher quantity of yeast.

The definite proof of the need to balance the protein in the diet is not possible of attainment with chicks. The experiments, however, indicate in a general way that with high protein the birds thrive better with a higher quantity of vitamin B as supplied in dried yeast.

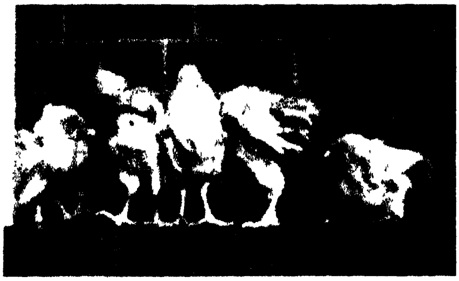
The photographs show the birds of groups XXXIII and XXXIV, the latter with the bow legs noticed with high protein in the diet (Plate VI, 1 and 2).



1
Group XXXIII

2
Group XXXIV

3
Group XXXVI



4
Group XLIV

5
Group XLVII A

6
(Group M VI p. 943).

II. THE BALANCE OF FAT BY VITAMIN B.

Our former observations that excess of cod-liver oil in the diet of chicks produced ill-effects and that these were counteracted by giving additional marmite were the starting-point of these experiments. Excess of cod-liver oil in the diet of chickens has also been found in agricultural experiments to be detrimental to their health and an endeavour was made to prove that vitamins were therefore of little value in nutrition. There was supposed to be an ample supply in the various foods given in agricultural practice.

In our experiments on high fat diets the use of a large quantity of cod-liver oil was avoided by working with cotton-seed oil. This oil does not contain any appreciable amount of vitamin A. Any ill-effect produced must therefore be attributed to the oil.

The diets for these experiments were planned on the same basis as those in the fourth series on protein balance. The experiments were carried out in succession as the results of each became clear. The series is shown in Table VI.

Table VI.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	Cotton-seed oil	No. of birds	Losses
XXXVI	58	12	2	8	20	15	0
XXXVIII	54	16	2	8	20	10	1
XLI	50	20	2	8	20	10	1
XLIV	60	10	2	8	20	8	5

Composition of the diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Ratio yeast/calories
XXXVI	8·1	2·8	0·2	13·8	22·6	52·2	481	1 : 40
XXXVIII	7·7	3·2	0·2	15·3	22·6	50·8	481	1 : 30
XLI	7·4	3·5	0·2	16·8	22·6	49·3	481	1 : 24
XLIV	8·3	2·7	0·2	13·1	22·6	52·9	481	1 : 48

Group XXXVI. Though there were no losses in this group, the birds looked abnormal throughout the time of the experiment, which lasted for 26 weeks, from 26. v. 1925 to 19. x. 1925. They were greasy to the touch and generally dirty in appearance. Slight leg weakness was noticeable in the 3rd week. It became more obvious in the 7th and 8th weeks and persisted until the end of the time. The birds were always sitting during the day. The chief feature of their appearance was the poor feathering; some birds were almost naked during the time of the trial.

Group XXXVIII. The birds of this group in contrast with those of the preceding one were always quite clean to look at and were not greasy to the touch. Their feathering was about as good as that of a normal bird. The one loss occurred in the 13th week. There was fluid in the body cavity and the intestines and appendices contained dark food material.

Group XLI. This group was tried to see if still more dried yeast would be of greater benefit in balancing the fat. No advantage was noticed. The loss in this group was in the 5th week and was due to pneumonia.

Group XLIV. The object of this experiment was to see the effect of less dried yeast than used in group XXXVI. The dirty and greasy appearance of the birds was again manifest. The first loss was in the 8th week. The heart was enlarged and covered with gelatinous matter. The appendices were swollen; the kidneys and liver pale. All the birds were very shaky at the 16th week and quivered as they stood. The second loss was in the 17th week; before death the bird showed distinct leg weakness. It was very fat in the abdominal region. The heart was also covered with fat. The appendices contained black food residues, and there was stoppage at their junction with the intestine.

The group was kept longer than the other groups to see the effect of long continuation of the diet. In the 23rd week the third loss occurred. The flesh of the bird was very wet, and there was fluid in the abdomen. It seemed as if there had been a burst blood vessel as blood clots were also noticed. The fourth bird died in the 26th week. It was also dropsical, its interior being full of gelatinous matter. The heart, kidneys and liver seemed very large and there were black food residues in the intestine. The fifth died in the 27th week. The inside was again very wet. Heart and liver were much enlarged. There was decomposing matter in the intestines and the appendices were swollen.

The other birds at the conclusion of the experiment on examination were more healthy in appearance. They showed a large amount of fat in the abdominal region and the heart of one was distinctly larger than normal.

There was a distinct difference in the rate of growth of the birds in these groups. It corresponded with the amount of dried yeast in the food, being greatest in group XLI and least in group XLIV. This could be attributed to the slightly greater amount of protein supplied in the food, but is more likely due to appetite which has so often been shown to depend on the quantity of vitamin B. The rate of growth is shown in Table VII.

The series of experiments clearly points to the need of balancing the fat in the food by vitamin B. The photographs of groups XXXVI and XLIV show the condition of the birds. The poor feathering of group XXXVI is not so clear in the picture as it was during the course of the experiment (Plate VI, figs. 3 and 4).

III. THE BALANCE OF CARBOHYDRATE BY VITAMIN B.

The experiment with group XXX in which extra white rice was included in the food in contrast with group XXIII had already shown that the whole of the carbohydrate needed balancing by dried yeast. In order to show the need of balancing more distinctly a series was started using the groups XXXIII and XXXV as the controls and feeding with diminishing quantities of dried yeast, until the amount was found which was not sufficient to rear the birds satisfactorily. The series is shown in Table VIII.

As stated above, group XXXIII was a perfectly normal group, and the losses in group XXXV have been described. It was not expected at the start that

Table VII. *Average weekly weights (g.) of birds in the balance of fat experiments.*

	Cocks				Hens			
Group	XXXVI	XXXVIII	XLI	XLIV	XXXVI	XXXVIII	XLI	LXIV
No. of birds	11	6	5	6	4	3	4	2
Week								
0	35	39	40	34	38	36	39	33
1	50	65	65	51	52	60	58	52
2	73	105	93	70	78	95	80	72
3	103	142	151	94	111	132	121	110
4	139	177	212	135	142	168	165	156
5	189	214	277	179	189	215	195	193
6	251	258	363	261	240	275	259	252
7	322	339	428	317	304	363	314	302
8	390	449	476	410	357	463	349	360
9	460	551	625	522	422	523	462	410
10	548	647	673	613	509	598	536	475
11	692	732	785	717	594	656	605	572
12	793	892	918	779	645	806	735	600
13	895	1058	1029	918	695	898	839	705
14	1030	1168	1260	999	811	972	999	762
15	1146	1310	1302	1092	900	1065	1054	785
16	1359	1515	1505	1208	1026	1233	1160	872
17	1462	1682	1723	1391	1114	1390	1317	715
18	1668	1852	1801	1581	1237	1497	1375	840
19	1880	1959	1986	1645	1341	1540	1477	850
20	2055	2111	2134	1802	1437	1605	1597	1000
21	2068	2215	2380	1870	1505	1735	1711	1030
22	—	—	2538	1993	—	—	1825	1100
23	—	—	2697	2136	—	—	1961	1200
24	—		—	2170	—	—	—	1245
25	—	—	—	2242	—	—	—	1360
26	—	—	—	2442	—	—	—	1435
27	—	—	—	2445	—	—	—	1585
28	—	—	—	2522	—	—	—	1655

Table VIII.

Group	White rice	Dried yeast	Cod-liver oil	Fishmeal	No. of birds	Losses
XXXIII	74	16	2	8	10	0
XXXV	78	12	2	8	10	2
XL	80	10	2	8	11	1
XLII	82	8	2	8	11	1
XLV	86	6	2	8	10	1
XLVI	86	4	2	8	13	0
XLVII A	88	2	2	8	12	2
XLVII B	88	2	2	8	9	6
L	89	1	2	8	5	5
XLIX	77	6	2	15	10	1

Composition of the diets %.

Group	Water	Ash	Fibre	Protein	Fat	Carbo-hydrate	Calories per 100 g.	Ratio yeast / calories
XXXIII	10·0	3·3	0·2	16·6	2·7	66·9	368	1 : 23
XXXV	10·4	3·0	0·2	15·1	2·7	68·4	367	1 : 31
XL	10·6	2·8	0·2	14·4	2·8	69·1	368	1 : 37
XLII	10·7	2·7	0·3	13·7	2·7	69·8	368	1 : 46
XLV	10·9	2·5	0·3	12·9	2·7	70·5	367	1 : 61
XLVI	11·1	2·4	0·3	12·2	2·6	71·3	367	1 : 92
XLVII	11·3	2·2	0·3	11·4	2·6	72·0	366	1 : 183
L	11·4	2·1	0·3	11·1	2·6	72·4	366	1 : 366
XLIX	11·1	3·8	0·2	16·7	2·9	64·9	362	1 : 60

so many groups would be required before the balance would be shown by losses of birds.

Group XL. Twenty-one weeks, from 10. vii. 1925 to 5. xii. 1925. One bird in the 9th week showed distinct leg weakness. It was given 1 g. of marmite and was cured next day, which showed that the leg weakness was due to lack of vitamin B. It never showed the symptoms again and was quite well at the end. The bird which died had an abscess under the skin which caused peritonitis. The excess cocks were killed in the 22nd week. Examination showed them to be quite normal in their appearance. One cock and the hens were then kept altogether for 52 weeks. They laid eggs, which were mostly unfertile. One hen died in the 42nd week. There were masses of fat in the body cavity, every organ being covered with fat. The connective tissue was leathery and the heart pale.

The group was considered quite normal throughout the experiment.

Group XLII. Twenty-two weeks, from 10. viii. 1925 to 11. i. 1926. The loss in this group took place in the 5th week. The bird had leg weakness and died suddenly. All the birds in the 8th week showed poor feathering and one had a head twist. They scattered their food. At the 18th week the birds looked pale and seemed to have signs of leg weakness. The excess cocks were killed in the 23rd week. All were very fat in the abdominal region and the appendices were full of pasty black food residues. The connective tissue was leathery. One cock and the hens were kept on longer. The cock died suddenly in the 31st week. It had a pale liver and large heart. The hens were put in with those of group XLV. The eggs laid were mostly unfertile, and the fertile ones did not hatch.

The group could not be considered as a perfectly normal one.

Group XLV. Twenty-one weeks, from 11. xi. 1925 to 7. iv. 1926. There was one loss in the 5th week. Hard lumps of food were found in the intestine and the heart was pale. The birds generally did not look as well as those observed in the previous groups and also scattered their food. The excess cocks were killed in the 22nd week. In all these birds the gizzards were flabby, but only small amounts of fat were noticed around the gizzards and hearts. The hens and one cock were kept on to the 38th week. Most of the eggs laid were unfertile; the fertile ones gave a few hatches but the chicks soon died, being in very poor condition.

The group was not a perfectly normal one.

Group XLVI. Twenty-one weeks, from 5. v. 1926 to 29. ix. 1926. Though there were no losses in this group, the birds that reached maturity were not normal. From the 3rd week the birds always left the rice of their food, but were not given fresh food until it was eaten. One bird in the 8th week had leg weakness, but got better. At the 20th week the birds looked very ill. Five showed distinct drooping of the wings and three had weak legs and were distorted to one side. They were killed in the 21st week. At *post mortem* examination the chief feature of all the birds was the enormous amount of fat around the necks and around all the internal organs. In many the hearts were enlarged. The intestines had pasty black food residues in them and the appendices were in some cases swollen.

The group was quite abnormal.

Group XLVII A. Twenty-one weeks, from 10. v. 1926 to 4. x. 1926. The first loss took place in the 14th week. The interior of the bird was very fatty and the appendices were slightly swollen. The heart, liver and kidneys were pale. The second loss was in the 17th week. The inside of the bird was very fat and the heart large. The birds in general did not feather well and were distinctly shaky on their legs. At *post mortem* examination the insides were found to be very fat, all the organs being more or less covered. In some the hearts were large and the intestines and appendices swollen. The birds were not by any means normal (see Plate VI, fig. 5).

Group XLVII B. Twenty-six weeks, from 29. x. 1926 to 29. iv. 1927. The survival of the birds of group XLVII A was so remarkable that a further experiment was made with the same diet. Six birds died during the course of 13 weeks. In each case the heart was large. In most the intestines had black food residues and the appendices in several were not normal. In these young birds there was not the visibly large amount of fat in the abdomen.

The three birds surviving to 26 weeks were hens. At *post mortem* examination they were found to be abnormally fat in the abdominal region. A mass of nearly 200 g. of fat could easily be removed by hand from one. They had large hearts and fluid was found in the pericardium of all three.

The losses and abnormality of the last three birds clearly show that 2 % of dried yeast is quite insufficient for balancing the food.

Group L. This group was started because the birds of group XLVII A lived so long. All birds except one died within 6 weeks. The single bird which survived lived for 20 weeks and then died suddenly. Its interior was not fat, but the small intestine was swollen and the heart large. The bird always ate very little and this may account for its long survival.

Group XLIX. Twenty-one weeks, from 6. viii. 1926 to 31. xii. 1926. This group was tried to see if more protein in the diet of the birds with 6 % of dried yeast, which were apparently normal in external appearance, would have any special effect. It was thought that high protein in the diet had a harmful effect. One bird died in the 3rd week from an unabsorbed yolk sac. The birds already in the 7th week looked ill and never seemed perfectly fit. They always panted after being caught for weighing. At *post mortem* examination some of the birds were found to be very fat. Several had crooked breastbones. The appendices generally contained pasty food and black food residues were often observed in the intestines. In many the hearts were obviously large. The birds were not normal, though there were no distinctive abnormalities. High protein appears to have an influence upon the health and appearance.

These experiments show definitely that 2 and 4 % dried yeast are insufficient to balance the carbohydrate in the food. With 6 % dried yeast the birds which reached maturity were not absolutely perfect. A really good result was only obtained with 10 % of dried yeast. The weights of the birds in the different groups as shown in Table IX also show quite definitely that 2 and 4 % are too little. The weights of the birds in the other groups, except possibly group XLII, are much the same each week. 6 % is the minimum of dried yeast and 10 % is a good quantity.

Table IX. *Average weekly weights of birds in the balance of carbohydrate experiments.*

Cocks

Group ...	XXXIII	XXXV	XL	XLII	XLV	XLVI	XLVII A
% of yeast	16	12	10	8	6	4	2
No. of birds	2	5	6	6	5	7	8
Week							
0	42	39	39	33	35	40	36
1	52	59	59	48	63	54	51
2	101	95	102	76	99	88	69
3	141	157	157	117	150	108	93
4	174	248	225	185	221	146	126
5	291	370	306	249	325	196	163
6	447	451	368	329	405	249	209
7	525	592	490	403	510	325	266
8	777	707	637	489	666	438	368
9	862	897	825	620	805	583	447
10	1122	1039	1002	743	1000	684	528
11	1317	1266	1156	963	1182	794	672
12	1522	1435	1343	1144	1390	1023	818
13	1730	1715	1533	1333	1479	1184	958
14	1955	1982	1758	1537	1695	1381	1103
15	2125	2140	1880	1779	1846	1584	1299
16	2335	2332	2207	1930	2139	1785	1508
17	2480	2589	2363	2119	2303	1979	1711
18	2655	2799	2584	2234	2498	2119	1889
19	2765	3074	2711	2412	2666	2240	2075
20	3080	3215	2955	2553	2684	2492	2248
21	3215	3225	3083	2778	2707	2359	2131

Hens

Group ...	XXXIII	XXXV	XL	XLII	XLV	XLVI	XLVII A
% of yeast	16	12	10	8	6	4	2
No. of birds	7	6	5	4	4	6	4
Week							
0	37	37	39	31	35	41	36
1	54	59	57	49	61	54	54
2	93	99	89	76	99	85	70
3	137	165	130	117	147	102	93
4	199	232	184	189	205	140	122
5	305	347	251	249	294	176	158
6	424	434	319	327	379	225	200
7	546	546	406	405	472	285	262
8	697	670	507	482	632	392	341
9	791	809	642	631	750	496	396
10	982	930	762	752	909	584	454
11	1141	1087	898	937	1061	695	554
12	1309	1202	1015	1076	1214	849	655
13	1441	1397	1154	1210	1300	977	754
14	1541	1532	1320	1387	1446	1133	848
15	1649	1607	1388	1541	1528	1287	952
16	1819	1678	1634	1624	1692	1444	1126
17	1964	1838	1720	1724	1771	1569	1290
18	2101	1944	1870	1844	1945	1681	1410
19	2164	2111	1963	1977	2109	1775	1531
20	2357	2217	2059	2045	2125	1932	1654
21	2457	2213	2120	2180	2420	1877	1660

EXPERIMENTS WITH PIGEONS.

During the course of the various trials with chickens similar experiments upon balancing the carbohydrate of the food with vitamin B were made with pigeons. Their diet was composed of white rice, later white flour, fishmeal and marmite, or dried yeast. Cod-liver oil was omitted, as it had previously been found that pigeons did not need cod-liver oil in their food [1923].

First series.

Two pigeons were placed on 1. i. 1924 upon the same diet as group XII 5, *i.e.*

White rice	Marmite	Fishmeal
90	9	5

At the end of 12 weeks it was discovered that they were two hens. A cock was substituted for one of them and the pair kept on this diet for 10 weeks. During this time two lots of two eggs were laid by the hen. The first lot was unfertile, the second was fertile and squabs were hatched but soon died. In order to improve the hatching and rearing, the quantity of marmite in the food was raised to 10 parts for the next 8 weeks, and then to 12 parts for a further 7 weeks. The eggs that were laid during these periods were deserted by the birds, one lot later disappeared, possibly eaten by the birds. As probably some other food constituent was needed for reproduction, a further increase in the marmite was not tried.

Altogether one bird was kept for 23 weeks and the other for 10 weeks with 9 parts of marmite and the two birds at the end showed a slight increase in weight. This quantity could therefore be considered ample for maintenance.

In order to find out the least quantity of marmite that would suffice to maintain the birds, the quantity was reduced to 4 parts. At the end of 6 weeks both birds had lost over 100 g. in weight and looked very ill. They had lost their appetite and scattered their food.

The marmite was raised to 6 parts; the food was now eaten without scattering and both birds increased in weight. It seemed that 6 parts of marmite was the quantity to balance the rice.

Our former figure for marmite in the same diet was 3 parts. The marmite was thus not so rich in vitamin B as in the former year. This was already indicated in the experiments with chickens on the balance of protein.

Samples of other yeast extracts.

This pair of pigeons, as seen above, were able to discriminate by scattering of the food and loss of appetite with concomitant loss of weight whether the food contained enough yeast extract or not. They were used for a rough test of several other samples of yeast extract which we were able to investigate.

The first sample, designated by us as *A*, was started at the level of 3 %. The birds lost weight and did not like the food. On raising the proportion to 3·5 % the birds recovered their appetite and weight.

The second sample, designated by us as *B*, was started at 3·5 %. The birds scattered the food and declined in weight. The amount was first raised to 4 %, then to 4·5, then to 5 and to 6 %. At this level the birds ate the food without scattering, regained their appetite and increased in weight. On again reducing to 5 %, they showed loss of appetite and loss of weight.

The third sample, designated *C*, was started at 5 %. The birds scattered the food and lost weight; on raising to 8 parts they recovered. On reducing to 7 they lost weight again, but recovered on raising to 10 %. The hen now suddenly died and the experiment was stopped.

The fourth sample of yeast extract, designated by us as *V*, was tried on another pair of birds. On 3 % the birds were kept for 11 weeks, and then for another 4 weeks at 4 % without any appreciable loss in weight.

The fifth sample of yeast extract, designated by us as *R*, was tested on a further pair of birds, starting at 1·5 % and raising the quantity to 3 % as they were not well; they died in 9 weeks. Another pair started at 4 % died in 7 weeks even after raising to 6 %. This extract thus appeared to contain very little vitamin B. The several extracts of yeast which have been so tested have varied from 3 or 3·5 to 8 or 10 % for the amount to be incorporated in a vitamin B-free diet. One extract was very weak.

Second series.

White rice soaked in marmite was not considered as an altogether suitable method of testing the value of yeast extracts, partly on account of the scattering of the food, and partly on account of the unavoidable loss of some of the extract adhering to the sides of the food containers which we used. It was decided therefore to substitute white flour for white rice. By making the flour into a dough with a solution of the marmite and making small pills about the size of a dried pea, drying the pills in the air, or at 37°, and feeding with such pills, it was impossible for the pigeons to separate the components of the food. It had to be swallowed whole, or left.

The marmite was then tested carefully at levels of 1 to 4 or more % until a quantity was reached which sufficed to maintain the birds for a long period. As seen below, the standard time of 15 weeks employed formerly by Chick and Hume [1917] is really not long enough and we have taken 20 or 26 weeks as a more satisfactory time for a maintenance test.

These experiments were made in succession as the result of a test became known. The birds on the smaller quantities showed the typical signs of polyneuritis and were allowed to die, proving the quantity was insufficient. Table X shows the results.

Table X.

Diet			
White flour	Fishmeal	Marmite	Result
90	5	2	Birds died in 7th and 10th weeks
90	5	4	„ 14th „ 20th „
90	5	5	„ 13th „ 16th „
90	5	6	„ 15th „ 18th „
90	5	8	„ 29th „ 36th „

On 2 parts of marmite the birds died with the typical symptoms of polyneuritis. One had an enlarged heart, the other showed no other special symptom.

On 4 parts of marmite, both birds showed polyneuritis. The second bird had these symptoms in the 16th week, but got better on being brought into a warm room. The symptoms appeared again in 4 weeks and the bird died. No special symptoms were noticed at autopsy.

On 5 % marmite, both birds showed polyneuritis. One bird had a distinctly large heart.

On 6 % marmite, polyneuritis was seen. The intestines were abnormal in appearance.

On 8 % marmite, the birds did not show the symptoms of polyneuritis. On *post mortem* examination the hearts were large and the intestines were abnormal.

The quantity of 8 % was taken as the minimum for long maintenance.

Third series.

It was also necessary to test the dried yeast used for the chickens as a source of vitamin B so as to compare it with the marmite.

It was being found that 6 % of dried yeast was a sufficient quantity to balance the carbohydrate for chickens. The first trial with pigeons was therefore made with 4 %.

The food was prepared as described above with white flour and made into pills.

On 4 % of dried yeast a pair of pigeons was kept for 37 weeks. During this time their weight kept constant and the hen laid six lots of eggs. The eggs were generally fertile, but only in two instances was a squab hatched; it died soon afterwards from unabsorbed yolk sac.

These birds were then given the diet with 2 % dried yeast.

They died in the 12th and 13th weeks afterwards. One bird showed the typical signs of polyneuritis; the other was paralysed. This latter bird had a distinctly large heart. The intestines of both birds were abnormal in appearance.

Other trials were made with 1 % and 3 % of dried yeast as these experiments were in progress. On less than 4 % the birds died. Still other experiments were made with larger amounts of dried yeast in the hope that rearing would be better. There was no great improvement in rearing, but the actual hatching was better. The results of this series are seen in Table XI.

Table XI.

Diet			
White flour	Fish-meal	Dried yeast	Results
94	5	1	Both birds died in 6 weeks
93	5	2	Birds died in 12th and 13th weeks
92	5	3	Birds died in 9th and 15th weeks
91	5	4	Kept for 37 weeks. Six lots of eggs, no rearing
89	5	6	Kept 18 weeks. Five lots of eggs; 2 hatches, no rearing. Then given 8 % dried yeast
89	5	6	Kept for 48 weeks. Eleven lots of eggs: fertile, but no hatch
87	5	8	Kept 50 weeks. Five lots of eggs: fertile; no hatching
85	5	10	Kept 26 weeks. Eight lots of eggs; three hatches, but no rearing

It was clear that 4 % of dried yeast was sufficient for long maintenance and reproduction. There was no rearing. The increase of dried yeast to 10 % made some improvement in hatching. The experiment was not continued, as it was possible that some other factor than vitamin B might be concerned in the hatching of young that could be reared.

The maintenance with 4 % of dried yeast is less than the amount with chickens, which were found to need 6 % as the minimum. These results correspond with those that we have found previously, that chicks need more vitamin B than pigeons. The larger amount of dried yeast in the food is beneficial just as was noticed with the rearing of chickens. For perfect health it is advisable to give more vitamin B than the minimum required for maintenance.

EXPERIMENTS WITH RATS.

Having found that 8 % of marmite and 4 % of dried yeast were necessary to balance the food of pigeons, it was of importance to ascertain how much vitamin B in similar terms was required by rats.

Exp. 1. This experiment, though not actually the first to be started, was made partly to act as control and partly to find out how long adult rats could live on a diet free, or almost free, from vitamin B. There are numerous references to the short time of survival of young rats on such a diet, but it is difficult to find information about adult rats.

Four adult rats, one buck and three does, which had been kept previously on a diet of 90 maize, 5 marmite and 5 fishmeal for 20 weeks were given our diet of:

White flour	Fishmeal	Cod-liver oil
94	5	1

made up into pills dried at room temperature on 4. vi. 1925.

Each animal showed an increase in weight during the first 4 weeks. One of the does then had young, which died. Subsequently a decline in weight set in. The other does also had young, which were eaten by their mothers. At the 8th week, a doe again had young which were eaten. In the 9th week another doe had young during the birth of which she died. The first doe then rapidly declined in weight and died in the 16th week. The third doe showed no signs of young.

The two remaining animals were now so ill that they were given a diet with 3 % of marmite. They showed improvement during the next few weeks, especially the buck, but they never seemed as well as our other rats. After 19 weeks on 3 % marmite in the food, both were chloroformed. The doe had seven young in her uterus, but her lungs were solid and she would have died of pneumonia. The buck was abnormal in appearance; the large intestine was yellow and contained gas; the appendices also contained gas. The animals did not appear to have recovered from the diet with no marmite.

During this period of diet with 3 % marmite, the buck was put with a doe also on 3 % marmite. This doe had been previously on 2 % marmite (see Exp. 2). At the 11th week on this diet this doe had young and she died of haemorrhage. On *post mortem* examination excess of fat in the abdominal region was the only abnormality observed.

Exp. 2. This was actually the first experiment on our white flour, fishmeal, marmite diet with 1 % of cod-liver oil. The amount of marmite was 4 %, half the amount that was found to be required by pigeons.

The object was to find out if a pair of rats could be maintained on this quantity of marmite and also reproduce. The experiment was started on 21. i. 1925 and the animals weighed 87 and 78 g. They increased in weight regularly every week. At the 6th week the doe had seven young which were eaten. At the 13th week she had a litter of nine. Five of these died, but the other four were reared by the mother and weaned in the usual time of 3 weeks (see Exp. 3).

At the 18th week the doe had another litter of nine; two of these were eaten.

As this doe had reared on 4 % marmite, the quantity was now reduced to 1 % to see if this quantity was sufficient for maintenance and rearing. The seven young gradually died, some showing distinct signs of paralysis. 1 % was thus not enough for rearing.

The two animals were then kept continuously on the diet with 1 % marmite. The doe had four more litters of young, which were at once eaten. The last litter was in the 34th week on this diet. During the birth of these young the doe died. Autopsy showed masses of fat in the abdominal region with black food residues in the large intestine extending into the small intestine. The buck rat did not look well and appeared very constipated; he passed only very few faecal pellets of very hard consistency daily. He lost weight, then gained about 40 g., then lost and then gained again. He was kept altogether 82 weeks on this diet and was then killed. Autopsy showed masses of fat in the abdominal region. Histological examination of the intestines of this rat has been made by Dr M. J. Rowlands who is publishing his results independently.

Exp. 3. The four young rats from the pair on 4 % marmite (Exp. 2) after weaning were given the diet with 3 % marmite for the next 3 weeks. During this time they increased in weight from 110 to 303 g., average 48 g. They were one buck and three does.

The quantity of marmite was now reduced to 2 % to see if this amount was enough for maintenance and reproduction. It had been seen that 1 % was not enough.

In the 7th week on this diet, one of the does gave birth to a litter of seven. Two were eaten at once, the others gradually died in the next 2 weeks. During the next 15 weeks each of the does had several litters of young. In every case the young were eaten by the mother. One doe was then changed to 3 % marmite (see Exp. 2). The other two does were kept on the 2 % diet for altogether 25 weeks. Both had young which they immediately ate. It thus seemed that 2 and 3 % marmite were not enough for rearing the young. The diet was changed to 4 % marmite, upon which quantity young had been reared (Exp. 2), for a further trial of this quantity. One doe had seven young which she ate. The other had ten, nine of which she reared (Exp. 4). The rats were kept on this 4 % diet for 16 weeks. Both does had young, but in each case there was no rearing. One of the does then died and at autopsy was found to have a ballooned intestine.

The trials with pigeons were now showing the superiority of dried yeast over marmite in vitamin B content. The marmite was changed to dried yeast. The pair was kept on 4 % dried yeast for 17 weeks. The young that were born were eaten in every instance by the mother. The dried yeast was raised to 6 % to see if a further increase would improve rearing. The buck died of pneumonia in the 25th week. The doe was kept for another 13 weeks and was given the buck alternately with the remaining doe from Exp. 4. No young were born. The experiment was then discontinued, as it was possible that reserves of a possible vitamin E were exhausted.

Exp. 4. In order to test again the 3 % quantity of marmite in the food, the nine young rats from 4 % (Exp. 3) were placed on this food after weaning. They increased from 26 to 74 g. on the average during the first 4 weeks. There were four bucks and four does and the ninth unfortunately escaped. Only one buck was kept and he was placed alternately with the four does, which were kept two in a cage except at birth of young.

Those on 3 % did not thrive well, only increasing 50 to 60 g. in the next 5 weeks. They were found one morning accidently injured and were chloroformed.

Those on 5 % marmite gained 70 g. in the first 5 weeks. One of these does also met with an accident during one night and was chloroformed. At this time the marmite was changed to dried yeast, as the marmite seemed less good than formerly.

A litter of five was born in the 18th week and two were reared. Though the pair was kept for another 40 weeks no more young were born. Altogether the pair was kept for 61 weeks on this diet.

Exp. 5. The two young rats from the 5 % dried yeast pair were two bucks. They were put upon a diet with 1 % dried yeast to compare with the diet of 1 % marmite. They were kept for 27 weeks and were then killed. On *post mortem* examination they were found abnormally fat in the abdominal area. Histological examination was carried out by Dr Rowlands who is recording his results separately.

Rats have thus been kept on diets containing 0, 1, 2, 3, 4 % of marmite. Adults were maintained for the long period of 34 and 82 weeks on 1 %, but young could not be reared. Young were not reared on 2 and 3 % marmite, but two litters were reared on 4 % marmite by different does. 4 % thus appears as the least possible quantity for proper maintenance.

Using dried yeast instead of marmite, young rats were kept for 27 weeks on 1 %, but on examination at autopsy they were abnormal. Reproduction was poor on 5 and 6 % dried yeast.

On comparison with pigeons, 4 % marmite for a rat corresponds with 8 % marmite for a pigeon and 2 % dried yeast with 4 % dried yeast, though rats could be maintained for a long period on 1 % dried yeast. Emmett and Peacock [1923] have also observed that rats have a smaller vitamin B requirement than pigeons.

A balance of food by vitamin B is thus also shown in the case of rats.

DISCUSSION OF RESULTS.

Though our experiments on the need of balancing the protein in the diet by vitamin B are not so conclusive as was hoped, there was no doubt that the chickens and ducks on the high amount of protein in the food were in better condition with additional marmite, or dried yeast. If our observations be taken in conjunction with those of Reader and Drummond [1926] and of Hartwell [1924, 1925] on rats, the conclusion may definitely be drawn that the protein of the food must be balanced by vitamin B. The ill-effect of the high protein on birds, not noticed on rats, is most likely due to the kind of protein. Reader and Drummond and Hartwell used caseinogen and in former experiments [1922] we have used a high proportion of milk proteins with which we reared first-class birds. It is probable that the flesh (fish) protein was the cause of the abnormalities in the birds.

The need for balancing the fat in the diet by vitamin B was made quite obvious in the miserable external appearance of the birds on the high fat diet with an insufficient amount of dried yeast.

The balance of the carbohydrate, though not so obviously affecting the external appearance, was shown by the abnormal *post mortem* condition of the birds at the end of the experiment.

Our results agree with those of Randoin and Simonnet [1924] who came to the idea that the ratio

$$\frac{\text{vitamin B}}{\text{carbohydrate}} = \text{a constant},$$

but our results point to a wider relationship, such as

$$\frac{\text{vitamin B}}{\text{calories}} = \text{a constant.}$$

If reference be made to the composition of our diets (pp. 923, 925) and if the ratio which we propose be considered, then in the high fat experiments, the ratio $\frac{\text{dried yeast}}{\text{calories}} = \frac{1}{40}$ and in the carbohydrate experiments the ratio is $\frac{1}{61}$, with 6 % of dried yeast, or $\frac{1}{37}$ with 10 % dried yeast. In the former case the yeast was scarcely sufficient, but the latter figure gave birds as good as those with 16 % dried yeast on the high fat diet.

A similar figure could not be arrived at from the protein experiments, as in all the experiments the ratio was greater than $\frac{1}{40}$.

In the case of pigeons the ratio is rather less, about $\frac{1}{80}$, and in the case of rats about $\frac{1}{160}$, if 2 % of dried yeast be taken as the minimum figure for their maintenance.

Cowgill, Smith and Beard [1925] and Beard [1925] have also found a relation between vitamin B and both food consumption and the body weight. They have proposed the relation

$$K = \frac{\text{vitamin B}}{\text{calories} \times \text{weight}^{\frac{5}{3}}}$$

and consider that during growth some additional factor affects the vitamin B requirement.

We have previously [1923] observed that young pigeons cannot be reared on the same diet as that on which their parents can be maintained. They need more vitamin B. The difference corresponded with the difference between adult pigeons and chicks. Young rats again could not be reared on the same diet as that on which their parents could be kept.

The function of vitamin B is more than that of simply balancing the food consumed and bearing a relation to the body weight. Drummond and Marrian [1926] have found that its rôle is not connected with the internal metabolism of the cell, for, in its absence from the food, the condition resembles the condition of the tissues in starvation.

Vitamin B appears to be of fundamental importance in life, having a rôle not only in general metabolism, but also a special rôle in relation to the endocrine organs. We believe that vitamin B is a definite and important chemical constituent of every cell, in fact a constituent of the nucleus. Without vitamin B the nucleus cannot exist. The greater abundance of vitamin B in nuclear tissues supports such a proposition, and, if Funk's original "vitamine" and other substances which have been isolated be accepted, the nuclear connection is emphasised by the fact that these substances belong to the pyrimidine or purine groups. The recent isolation of the antiberiberi vitamin by Jansen and Donath [1927] again includes the vitamin in this group of compounds.

The function of protein in the food is to repair so-called wear and tear of the cell and for growth in young animals. Similarly, the function of vitamin B is to replace wear and tear of the nucleus and to aid the growth of nuclear material. Hence the greater need of vitamin B by young animals, for growth of the nuclei. The difference in requirements between birds and rats may be in connection with the amount of nuclear material in the whole body. Rats may have a relatively smaller amount of nuclear matter for repair and for growth.

Since it is clear that vitamin B must bear a relation to the total food consumed, it would follow that the general idea of a daily dose or quantity is not correct. Such a quantity might be determined for a small amount of daily food, but this quantity would not suffice for a larger amount. The quantity of vitamin B required daily must be in relation to the maximum daily food consumption.

Our experiments further make a distinction between absence of vitamin B, an insufficiency and a proper amount. The term deficiency has been generally used to describe the condition of total, or nearly complete, absence. The term partial deficiency has been used for amounts between absence and the proper amount. We propose the term *shortage* for the intermediate condition of too little, more particularly to a slight degree which produces no immediate ill-effects.

Very definite pathological symptoms have been noticed in our animals kept for long periods on a shortage of vitamin B. The very large amount of fat in the abdomen was the most striking condition. The large intestine was often blocked with dark residues of food, occasionally extending into the small intestine. Sometimes there was stoppage in the large intestine, as was noticed by the impossibility of pushing the food down the alimentary canal. The pigeons often had the cloaca distended with excreta. It has been noticed also that after evacuation pigeons with polyneuritis were distinctly better. The appendices were abnormal. The hearts in many cases were obviously enlarged. The tissues were sometimes wet. The gizzards in some cases had very thin walls. Unfortunately we have not been able to study the pathology of the organs, but Dr Rowlands is referring to the histology of the intestines in cases of shortage in rats, and Dr Pulvertaft has examined many of the hearts in our dead birds.

The pathological appearances which we have noticed are the same as described by McCarrison [1921] as occurring in the early stages of beriberi, in which vitamin B is absent from the diet. The early stages of beriberi have thus become a chronic condition on a diet with a continual slight shortage of vitamin B. They are the conditions commonly observed in the population at the present time. For this reason we have also made tests of the comparative vitamin B value of the common foods, some of which we hope to have ready for publication shortly. These tests indicate that the general diet of the people is like that of the birds on a slight shortage of vitamin B, and that the symptoms produced by vitamin B shortage are the same in birds and man. The prevalence of gastro-intestinal trouble and heart affections may be traced to a continual slight shortage of vitamin B.

One other factor may be considered as concerned in the pathological condition of our animals, namely, the fat-soluble vitamin D. Though 2 % cod-liver oil was fed to all the chickens in the last series of experiments and rats to supply fat-soluble vitamins, it is possible that owing to faulty digestion on account of shortage of vitamin B the oil was not assimilated, so that there was at the same time a shortage of vitamin D. A full supply of vitamin B is essential for the introduction into the system of the vitamins A and D in the fat of the food.

Our results and conclusions are based upon the old idea that marmite and dried yeast contained only one vitamin B. Two vitamins, which may be termed B 1 and B 2, are now believed to be present in these foods. It is therefore possible that both B 1 and B 2 are concerned, or possibly B 2 only. The future will have to decide upon the separation and differentiation of these vitamins. For practical purposes, at any rate of human dieting, the existence of the two vitamins, closely associated in yeast and other foods, is in the same category as the existence of two fat-soluble vitamins, A and D in cod-liver oil, butter, other fats and eggs. The foods which supply the one most often contain the other also, so that they are consumed together.

SUMMARY.

Protein, fat and carbohydrate in the diet need balancing by vitamin B (B 1 + B 2). The quantity of dried yeast, which carries the vitamin B required, can be represented by

$$\frac{\text{dried yeast}}{\text{calories}} = \frac{1}{40} \text{ to } \frac{1}{60}$$

or from 6 to 10 % in a food containing no vitamin B in the case of chickens. The ratio is about $\frac{1}{80}$, or 4 % of the food, in the case of adult pigeons and $\frac{1}{160}$, or 2 %, in the case of adult rats.

8 % of marmite corresponded with 4 % of dried yeast as tested on pigeons. Samples of yeast extract have been found to vary considerably in their vitamin B content.

Young animals require more vitamin B than adult animals. It is therefore suggested that vitamin B is a constituent of the nucleus of the cell, and is required for growth and for replacement of wear and tear.

Various pathological conditions, obviously affecting the intestines and heart are noticed on diets containing too little, or a shortage, of vitamin B. These symptoms have been described as occurring in the early stages of beriberi. On a shortage of vitamin B these early symptoms become a chronic condition of ill-health.

We gratefully acknowledge a grant from the Government Grant Committee of the Royal Society, which has covered a portion of the expenses of these experiments. We also wish to thank the Marmite Food Extract Co., Ltd., for kindly placing at our disposal marmite and dried yeast.

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CXXVI. EXPERIMENTS ON NUTRITION. VII.

FAT-SOLUBLE VITAMIN REQUIREMENTS OF CHICKENS.

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(*Received June 14th, 1927.*)

OUR original objects in the study of the rearing of chickens were to ascertain the minimum quantities of each vitamin, in terms of cod-liver oil, marmite or yeast, and orange juice, that were required [1922]. We have found [1923, 1] that vitamin C is not required, that the vitamin B requirement is 9 % of marmite [1923, 2], or 10 % of dried yeast [1927]. The quantity of fat-soluble vitamin in terms of cod-liver oil has in the meantime been determined by Dunn [1924] at 0·5 %. Emmett and Peacock [1923] have also referred to the requirement of vitamin A by chickens and contrasted their requirement with that of pigeons and rats. Pigeons need very little, if any, and rats were thought to need less than chickens.

It became necessary for us, in connection with our work on the balance of food by vitamin B, to ascertain the amount of fat-soluble vitamins required, since a possible cause for the bow legs and other deformities observed on a high protein diet was little cod-liver oil in the diet.

EXPERIMENTAL.

These trials have been made during the course of the previous experiments.

The conditions of rearing were the same as then described.

All the cod-liver oil has been obtained from the same source and said to be of the same quality. It was procured in 1 or 2 gallon lots at different times and may not always have contained the same amount of fat-soluble vitamins. This may account for slightly different results with approximately the same quantity of the oil. It was not possible to test each lot for its vitamin content by special experiments.

A series of experiments, as shown in Table I, has been made.

The first three experiments were made in the same fashion as the first three series with high protein (see foregoing paper), in which one part of cod-liver oil was made up with the food. Group XXIII is the corresponding one to those in this series. The last five experiments were made with a diminishing percentage of cod-liver oil. Groups XXXIII and XXXV are controls.

Table I.

Group	White rice	Dried yeast	Fishmeal	Cod-liver oil	No. of birds	Losses
XXIV	90	20	5	0	8	8
XXXI A	90	20	5	0·5	3	2
XXXII A	90	20	5	0·7	8	4
		or				
XXIV	78	17	4	0		
XXXI A	78	17	4	0·4		
XXXII A	78	17	4	0·6		
XXXIX	75·3	16	8	0·7	8	0
XLIII	75·5	16	8	0·5	9	0
XXXI B	75·7	16	8	0·3	8	1
XXXII B	75·8	16	8	0·2	8	1
XLVIII	75·9	16	8	0·1	7	2

Group XXIV. All the birds died within 4 weeks. Autopsy showed that the bones were soft. The intestines showed no special abnormality, but the kidneys were generally pale in colour. In one bird the body cavity was full of water.

Group XXXI A. The birds were clumsy on their legs in the 3rd week; the legs were stretched outwards rather than twisted as in the abnormalities on the high protein diets. The two losses were in the 4th and 5th weeks. The yolk sacs were found not to be completely absorbed. The livers were not normal and the kidneys seemed distended. The excretion of these birds seemed difficult. The remaining bird was then placed in the group XXXII A.

Group XXXII A. The four losses occurred in the 3rd and 4th weeks. All these birds showed some congestion round the intestines; the guts were clean. The lungs of all were congested and they died of pleurisy. The other birds seemed fairly normal. The experiment was not continued further, as it already indicated that 0·6 part of oil was hardly sufficient. The early deaths of these birds in contrast with the longer life of the birds in the protein series is remarkable. The slightly larger quantity of cod-liver oil in those experiments was at any rate beneficial, though it may not have been enough. The later experiments were made to compare with the fourth series of protein experiments and the series with high fat and carbohydrate.

Groups XXXIX *and* XLIII. The birds in these two groups were probably the best birds that were reared in any of our experiments. Those of group XLIII with one cock were kept altogether for about 40 weeks and never showed any signs of illness, or deformity. They started laying in the 24th week. Many of the first eggs were soft, and the birds were egg eaters. On testing for fertility by incubation the eggs were mostly unfertile.

0·5 % of cod-liver oil of good quality is sufficient to rear chicks to maturity.

The other experiments in this series were made to find out how far the quantity of cod-liver oil could be reduced.

Group XXXI B with 0·3 % of oil gave a good lot of birds with only one loss. This bird was weak on its legs in the 12th week and was killed in the 19th week. There was no fat inside, but the appendices were slightly swollen; no sign of gut stoppage could be found. The gut was practically empty; the heart was possibly a little larger than normal. Examination of the birds at the end of the experiment when they were killed showed the intestines to be practically normal; the appendices were not quite normal in all the birds; in one case there was dark food material in the appendices. The hearts seemed slightly large.

Group XXXII B. The one loss was in the 9th week. This bird was weak on its legs and was chloroformed. It had a large wound in one leg, probably from injury. No abnormality could be seen internally. Two of the birds had deformed wings, but no special error could be detected in the internal organs after death. There was a little fat round the gizzards, in one case there was fat round the heart.

Group XLVIII. The first loss was in the 5th week. The kidneys were very pale. The appendices were distended and there was blood on the food residues in them. The rest of the gut seemed normal. The other loss was in the 15th week. This bird was shaky for the previous 2 weeks and had to be killed. The intestines seemed normal, though dark food material was present in the appendices.

It was remarkable that the birds could be reared on such small quantities of cod-liver oil as 0·1 and 0·2 %. In appearance, however, the birds could not be said to be quite fit and well.

A comparison of the weekly weights (g.) of the different groups, given in Table II, shows that the rate of growth is almost the same in the several groups. The cocks grew more slowly on quantities of cod-liver oil below 0·5 %, as was made evident first at the 10th and 11th weeks.

Table II.

	Cocks				
Group ...	XLVIII	XXXII B	XXXI B	XLIII	XXXIX
Cod-liver oil %	0·1	0·2	0·3	0·5	0·7
Week					
0	41	38	38	41	39
1	73	56	54	61	68
2	117	82	86	102	128
3	183	148	140	176	211
4	272	236	192	215	299
5	325	348	273	315	384
6	407	502	357	442	502
7	550	653	461	552	623
8	678	810	650	726	827
9	833	993	847	956	963
10	995	1085	957	1135	1181
11	1155	1290	1231	1362	1401
12	1388	1485	1363	1621	1682
13	1527	1540	1611	1817	1856
14	1755	1847	1808	2074	2105
15	2082	2010	1988	2314	2304
16	2068	2235	2185	2520	2586
17	2372	2505	2249	2794	2730
18	2618	2547	2537	2794	2910
19	2892	2805	2735	3066	3045
20	2972	2800	2974	3007	3348
21	3075	2745	—	3112	3485

Hens

Group ...	XLVIII	XXXII B	XXXI B	XLIII	XXXIX
Cod-liver oil %	0·1	0·2	0·3	0·5	0·7
Week					
0	40	39	39	37	38
1	71	52	55	56	70
2	119	78	88	92	117
3	182	124	137	154	175
4	294	187	185	187	243
5	298	269	275	260	305
6	383	361	357	372	402
7	490	476	445	477	500
8	593	588	610	615	637
9	732	745	790	796	768
10	865	855	878	939	915
11	1033	1009	1085	1079	1050
12	1233	1178	1162	1263	1238
13	1373	1275	1330	1404	1300
14	1622	1501	1432	1549	1455
15	1687	1587	1548	1684	1578
16	1712	1765	1690	1810	1737
17	1872	1903	1748	1950	1807
18	2025	1914	1960	1954	1927
19	2173	2117	2095	2161	2000
20	2236	2177	2130	2142	2122
21	2370	2030	—	2259	2220

The earlier experiments showed that 0·4 and 0·6 % of cod-liver oil were too little. The later experiments showed that 0·5 % was enough. The actual quantity depends upon the actual value of the cod-liver oil. In general it may be said that 1 % will be an ample quantity.

Experiments on a maize diet.

Some work in another direction using maize as a source of carbohydrate and vitamin B has led to another test regarding the requirement of cod-liver oil in the diet. There have been three groups tried so far for this purpose.

Diet.

Group	Maize	Fishmeal	Cod-liver oil	No. of birds	Losses
M V	89	10	1	8	0
M VI	90	10	0	9	5
M VII	89·75	10	0·25	6	1

M V birds have been reared successfully.

M VI birds showed definitely in the first 4 weeks symptoms of rickets and five died, showing congested intestines and appendices with stiff pasty food. These birds are shown in Plate VI, fig. 6.

Their appearance is quite different from that of the birds on high protein diet. Their legs are stretched outwards. The high protein birds had either bow legs or were knock-kneed. Often also the birds with too little vitamin B had a twisted leg. The outstretching seems to be the sign of rickets in chicks. In the early symptoms of polyneuritis the bird tends rather to sit on its legs and one leg tends to bend underneath the bird.

M VII birds, except one, have lived for 8 weeks and up to that time have shown no signs of rickets in their legs. Signs have then appeared.

It is thus essential to add cod-liver oil to the maize diet to rear chicks, and 0·25 % is too little.

Just as in the rice-dried yeast birds the minimum quantity of cod-liver oil appears to be 0·5 %.

We have several times observed that on keeping groups of birds beyond maturity these small quantities of cod-liver oil are not enough. The eggs have been unfertile and if fertile did not hatch, or the chicks soon afterwards died. On raising the cod-liver oil to 3 % improvement in fertility and hatching was observed. It is proposed at a later date to test this point specially.

SUMMARY.

The quantity of cod-liver oil that is required for rearing chicks is 0·5 % as a minimum. Samples of cod-liver oil, presumably of the same quality, vary and it is better to take 1 % as the smallest quantity.

Part of the expense of this work has been defrayed out of a grant to one of us from the Government Grant Committee of the Royal Society, to whom we express our thanks. Dried yeast has been kindly placed at our disposal by the Marmite Food Extract Co., Ltd.

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CXXVII. THE DIURNAL VARIATION OF THE GASEOUS CONSTITUENTS OF RIVER WATERS.

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HISTORICAL INTRODUCTION.

MORREN [1841] studied the variations of dissolved oxygen in an aquarium throughout almost a year and made observations of diurnal changes of the oxygen content of the water. His figures, however, record the percentage oxygen in the total dissolved gases, and he makes only one reference to the actual amount present. He says: "Le volume de l'air dissous était variable et augmentait notablement avec la quantité d'oxygène. La quantité d'azote restait sensiblement constante mais l'acide carbonique contenu dans l'eau variait aussi." He gives no figures of actual quantity present, but it is of interest that he records supersaturation.

In rivers, however, he finds no such variation. He suggests that his failure to detect a maximum is due to a rapid current, and a sandy bed, and without giving any figures says that a slow river flowing over mud does not contain so much dissolved oxygen, and that the figures obtained show considerable variation.

Considerable work has been done on the seasonal variations of dissolved gases in lakes and reservoirs, and on the relation of the dissolved gases to the presence of algae.

Birge and Juday [1911] have recorded the dissolved oxygen content of Lake Mendota at various depths over a period of 12 months, but showed no diurnal variation.

Butler and Coste [1923], working in the Thames estuary, showed seasonal variation of dissolved oxygen, and commented on the phenomenon of supersaturation.

The relation of dissolved gases to algae has been demonstrated by Chambers [1912] on the results of his work in the lagoons at the botanical gardens of St Louis; of special interest is his observation that on clear sunny days the water became supersaturated with dissolved oxygen, while on cloudy days the percentage of oxygen fell below saturation, sometimes as low as 40 %.

Moore [1921], correlating the results of 10 years' work, showed the seasonal variations and the interrelations of dissolved oxygen, diatoms, light, temperature, nitrite and alkalinity in the sea.

The present paper giving the diurnal variation of dissolved gases in small rivers is an addition to the data there recorded.

Cowles and Schwitalla [1923] have recorded some similar variations in hydrogen ion concentration, but no figures have been given for diurnal variation in ammoniacal nitrogen.

OBSERVATIONS ON RIVER WATERS.

During the past year several surveys have been made of the River Lark in connection with the pollution by the effluents from the beet-sugar factory at Bury St Edmunds. The River Lark is a small river rising in the chalk hills to the south of Bury St Edmunds. From here it flows in a north-westerly direction and joins the Great Ouse near Prickwillow, 25 miles from the source.

The beet-sugar factory at Bury St Edmunds discharges its waste waters into the river about a mile north of the city, and the part of the river under observation stretches from Bury through the villages of Fornham, Lackford, Icklingham, Barton Mills, Mildenhall to Westrow, a distance of 14 miles. The usual determinations made on these surveys were:

1. Dissolved oxygen.
2. Ammoniacal nitrogen.
3. p_{H} value.
4. Water temperature.

It was noticed that occasional anomalies occurred in the values obtained from the dissolved oxygen determinations, *e.g.*

At Icklingham, July 29th, 1926, 10.20 a.m., 59 % of saturation of O_2.
2.5 p.m., 105 ,, ,,
At Fornham, March 22nd, 1927, 3.35 p.m., 112 % of saturation of O_2.
23rd, 1927, 4.20 p.m., 88 ,, ,,
24th, 1927, 12.20 p.m., 118 ,, ,,
25th, 1927, 11.20 p.m., 90 ,, ,,

and it was concluded that such factors as time of sampling, weather conditions, amount of light, etc., have a greater effect upon the amount of dissolved oxygen present than was previously supposed.

RIVER LARK. SERIES I.

Accordingly, it was decided to carry out hourly determinations of dissolved oxygen, ammoniacal nitrogen, p_{H} and water temperature, over a period of 24 hours at a fixed point.

A convenient place for sampling was chosen at Icklingham, 7 miles below the confluence of the river and the leet from the beet-sugar factory.

The figures obtained over a period of 10 months are given below to indicate the changes produced at this spot during the beet-sugar campaign.

Table I.

Date	Time	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation of oxygen	p_H value	Ammoniacal nitrogen. Parts per 100,000	Remarks
29. vii. 1926	10.20 a.m.	15	0·59	59	7·5	0·05	
29. vii. 1926	2.5 p.m.	17	1·01	105	—	—	
14. ix. 1926	7.20 p.m.	15	1·05	102	8·2	0·02	
6. x. 1926	—	14·5	—	86	8·0	0·01	Fermented effluent discharged from factory
27. x. 1926	5.15 p.m.	6	0·395	31	7·5	0·02	Unfermented effluent discharging from factory
28. x. 1926	2.5 p.m.	6	0·485	38	7·5	0·02	
29. x. 1926	10.15 a.m.	7	0·203	17	7·4	0·15	
16. xi. 1926	3.10 p.m.	9·5	0·096	8	7·3	0·01	Process water and wash water discharging from factory
17. xi. 1926	2.10 p.m.	10	0·090	8	7·3	0·015	
23. ii. 1927	5.45 p.m.	9	0·702	60	7·6	0·03	Effluent ceased, but pits being washed out
24. ii. 1927	9.45 a.m.	8	0·602	52	7·6	0·08	
25. ii. 1927	4.45 p.m.	6	0·845	67	7·8	0·02	Full flood
7. iii. 1927	5.45 p.m.	7	1·021	83	7·7	0·04	
8. iii. 1927	4.35 p.m.	8	1·098	91	7·8	0·035	
10. iii. 1927	1.45 p.m.	6·5	1·112	90	7·7	0·04	
22. iii. 1927	2.15 p.m.	12	1·588	144	7·8	0·02	
23. iii. 1927	3.10 p.m.	12	1·555	141	8·0	0·015–0·02	
25. iii. 1927	12.15 p.m.	8	1·221	102	7·8	0·03	
26. iv. 1927	11.40 a.m.	8·75	1·354	118	7·7	0·035	

From the above figures it will be seen that, at this spot during the months of October and November, 1926, the river was badly de-oxygenated when the various effluents were being discharged from the factory, and that, after February, 1927, when the beet-sugar campaign ended, the recovery in oxygenation was rapid.

As a result of the October and November pollution, the whole bed of the river over a stretch of 10 miles had been covered with a growth of *Sphaerotilus natans*, a sewage fungus, which in February, 1927, commenced to turn brown, decay and break up.

By March 11th, the sewage fungus had almost disappeared and on the date of the experiment, March 30/31st, large quantities of diatoms had grown on the weeds and river bottom (see below). At Icklingham, the river is about 30 feet wide, and has an average depth of 3–4 feet, and 200 yards below the point of sampling is a mill where the river is dammed. The speed of flow is about 600 yards per hour.

The period of observation extended from 7 p.m., March 30th to 7 p.m., March 31st; hourly samples were taken, and the following determinations made:

1. Dissolved oxygen (Winkler's method).
2. Ammoniacal nitrogen (by direct nesslerising and subsequent comparison against artificial colour standards).
3. p_H value. (On the River Lark, by addition of cresol red or phenol red and comparison against Clarke's colour chart. On the River Itchen, by Gillespie's method.)

4. Water temperature.

The results obtained are given in Table II and graphically in Fig. 1.

Table II.

Time G.M.T. Mar. 30th, 1927	Temp. ° C.	Dissolved oxygen. Parts per 100,000		% saturation dissolved oxygen		Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
		Without prelimin. oxidation	With prelimin. oxidation	Without prelimin. oxidation	With prelimin. oxidation			
7 p.m	9·4	1·384	1·377	119	119	0·01–0·015	7·9	Fine
8 "	9·2	1·260	1·249	108	107	0·015	7·9	"
9 "	9·2	1·147	1·127	98·5	97	0·02	7·9	"
10 "	8·6	1·036	1·017	88	86	0·025–0·03	7·9	"
11 "	8·0	0·972	0·711	81	59	0·03–0·035	7·8–7·9	"
Midnight	8·0	0·865	0·846	72	70·5	0·11	7·7–7·8	
Mar. 31st, 1927								
1 a.m.	7·7	0·798	0·784	66	65	0·13	7·7–7·8	
2 "	7·1	0·797	0·796	65	65	0·16–0·17	7·6–7·7	
3 "	7·1	0·795	0·694	65	56	0·15	7·6–7·7	
4 "	7·0	0·800	0·805	65	65	0·15–0·16	7·6–7·7	
5 "	6·75	0·806	0·791	65	64	0·16	7·6–7·7	Dawn
6 "	6·9	0·808	0·769	66	63	0·13–0·14	7·6–7·7	Bright
7 "	6·9	0·810	0·783	66	63	0·1	7·6–7·7	Bright sunlight
8 "	6·9	0·838	0·861	68	70	0·07	7·6–7·7	"
9 "	7·0	0·953	0·920	77	75	0·06	7·6–7·7	"
10 "	7·5	1·094	1·089	90	90	0·07–0·08	7·7	"
11 "	8·2	1·413	1·405	119	118	0·03	7·8	"
Midday	8·9	1·535	1·501	131	129	0·02	7·8–7·9	Dull
1 p.m.	9·25	1·679	1·646	144	141	0·01–0·015	7·9	"
2 "	9·5	1·758	1·692	151	146	0·01–0·015	7·9–8·0	"
3 "	10·0	1·803	1·819	157	158	0·01	7·9–8·0	"
4 "	10·0	1·767	1·726	153	150	0·01	7·9–8·0	"
5 "	9·75	1·690	1·614	146	139	0·01	7·9–8·0	Rain
6 "	9·6	1·513	1·467	130	126	0·01	7·9–8·0	"
7 "	9·25	1·343	1·270	116	109	0·01–0·015	7·9–8·0	Sunset

Sunset March 30th, 1927, 6.28 p.m.
Sunrise " 31st, " 5.40 a.m.
Sunset " 31st, " 6.29 p.m.

Variation of dissolved oxygen.

The dissolved oxygen content of the river showed a remarkable periodic variation between 0·795 and 1·803 parts per 100,000, *i.e.* between 65 and 157 % of saturation of oxygen at the temperature of sampling.

From 7 p.m., March 30th, the values obtained decreased steadily until 1 a.m., March 31st, from 1 a.m. to 7 a.m. the dissolved oxygen content was at a minimum, after which a very rapid increase took place, reaching the maximum value at 3 p.m.; from 3 p.m. onwards, a rapid decrease took place for the remainder of the period of observation.

From 9 p.m., March 30th, until 10 a.m., March 31st, all samples had a dissolved oxygen content below 100 % of saturation, while for the remainder of the 24 hours the water was supersaturated.

The samples which underwent preliminary oxidation before estimation of dissolved oxygen generally gave values about 2 % less than those fixed and estimated in the ordinary way.

Variation of ammoniacal nitrogen.

Considering the fact that the ammoniacal nitrogen content of a normal river water is about 0·01 parts per 100,000, the figures obtained during this experiment must be regarded as abnormal, and it may here be pointed out that very high ammonia figures have been obtained at various stations on this river, coincident with the presence of large quantities of broken and decaying sewage fungus.

At 7 p.m., March 30th, the water contained 0·01–0·015 parts N per 100,000, which increased to 0·015 parts at 8 p.m. From 8 p.m. until 11 p.m. the increase in the ammoniacal nitrogen content was more rapid, and after 11 p.m. it increased still more rapidly, reaching a maximum value of 0·165 parts per 100,000 at 2 a.m., March 31st. and remaining at about this figure until 5 a.m.

After 5 a.m. the values obtained fell very rapidly until 1 p.m.; the minimum value of 0·01 parts per 100,000 was reached at 3 p.m. and this figure was maintained until 6 p.m., followed by a slight increase at 7 p.m.

Variation of p_H value.

During the period of observation the reaction of the water changed slightly between p_H 7·6 and 8·0.

After 10 p.m., March 30th, the p_H value decreased gradually, reaching a minimum at 2 a.m., March 31st, and remaining constant until 9 a.m., after which a steady increase took place to a maximum value at 2 p.m. which persisted until 7 p.m.

Variation of water temperature.

From 7 p.m., March 30th onwards, the water temperature fell steadily, reaching a minimum of 6·75° at 5 a.m., March 31st. After 10 a.m. it increased constantly to a maximum of 10° at 3 p.m., after which a steady decrease took place. The range was from 6·75° to 10°.

Conclusions based on the chemical data.

It is well known that all green plants under the influence of light consume carbon dioxide and liberate oxygen. On the date of the experiment, the river contained very few of the larger green plants, but diatoms were at the spring maximum of their growth. The result is seen in the abnormal figures of supersaturation of dissolved oxygen obtained during the experiment. Large clots of these organisms, floating in the water, gave evidence of their abundance. Rough estimations of this growth have been made by putting glass slides on the river bed. The slides are each 2·5 × 8·2 cm. and four are fixed in an ordinary photographic printing frame. These are taken up after a fortnight's immersion, washed with distilled water and the deposit dried at 100° and weighed.

	Amount in g. per sq. cm.	
Dates of immersion	No. 1 frame	No. 2 frame
1. xi. 1926 to 15. xi. 1926	0·003	0·004
9. iii. 1927 to 23. iii. 1927	0·010	0·015
23. iii. 1927 to 7. iv. 1927	0·073	0·070
7. iv. 1927 to 21. iv. 1927	0·047	—

Animals and fungi, as well as green plants, take up oxygen during both day and night and give out carbon dioxide. During the night, photosynthetic production of oxygen ceases, while biological and chemical consumption of oxygen continues, so that the dissolved oxygen content of the water is at its minimum during this period.

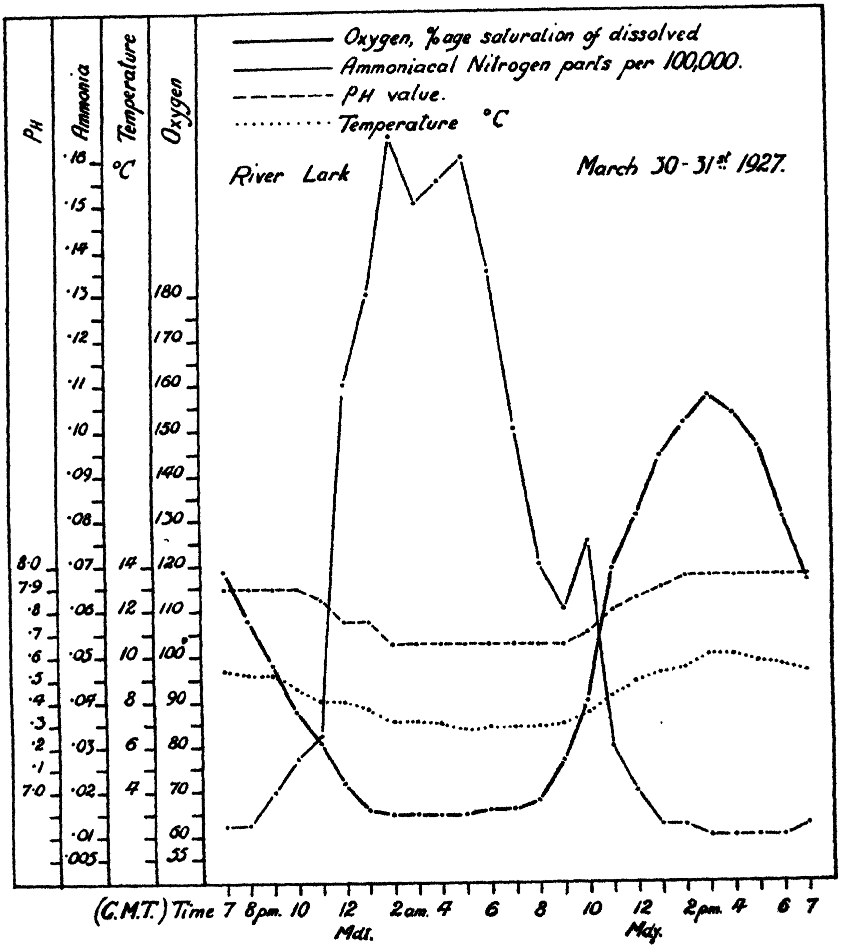

Fig. 1.

At Lackford (2 miles above Icklingham) a small sewage effluent enters the river with an ammoniacal nitrogen content of 0·8 parts per 100,000. Previous samples, taken during the day, 400 yards below the confluence of the leet and river, show that the dilution is sufficient to reduce the nitrogen content to 0·01 parts per 100,000.

By the decay of vegetable matter a considerable amount of ammonia is produced; some figures, obtained on previous surveys, are given below showing very high ammonia values coincident with the presence of large quantities of broken sewage fungus.

	N parts per 100,000
Feb. 23rd, 1927, Barton Mills	0·095
„ 23rd, 1927, Mildenhall	0·135
„ 23rd, 1927, Westrow	0·125
Mar. 7th–10th, 1927, Fornham to Westrow (15 miles)	0·03–0·15
„ 22nd, 1927, Barton Mills	0·16

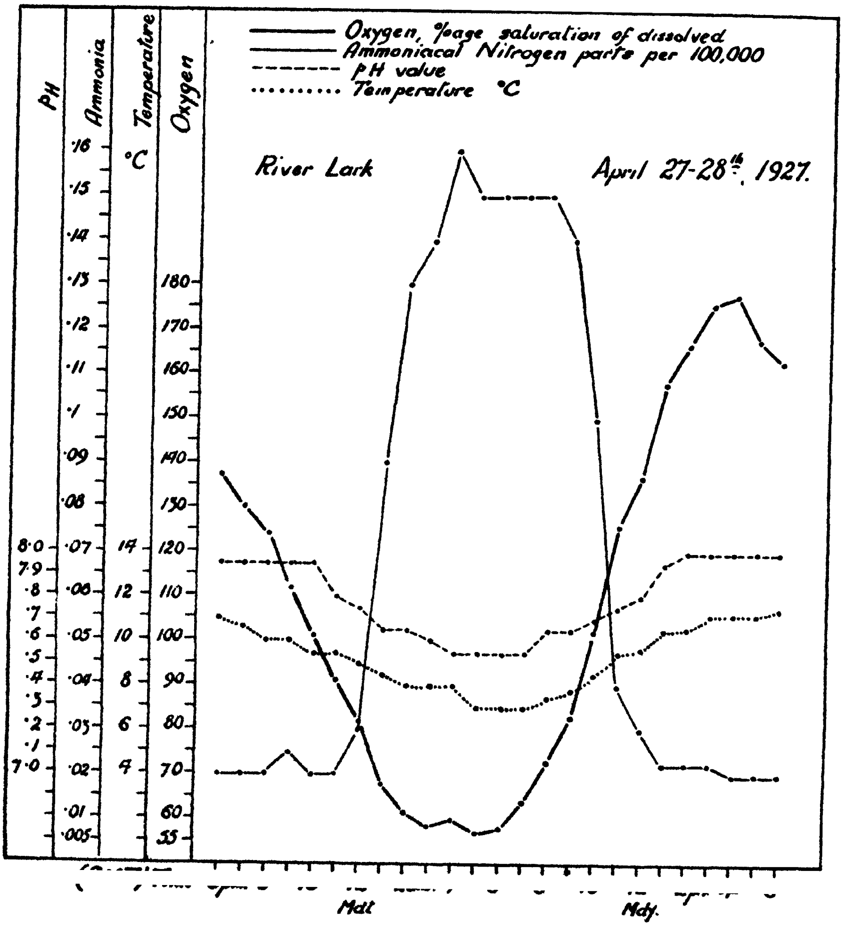

Fig. 2.

During the day, when the oxygen content of the river is high, the ammonia is oxidised to nitrates, with the result that after 3 p.m., when the oxygen content is at a maximum, the ammoniacal nitrogen content is at a minimum [cf. Bonazzi, 1919]. Correspondingly, when the oxygen content has fallen below 100 % of saturation, less of the ammonia produced is removed by oxidation, and during the minimum of oxygen period, 1 a.m. to 7 a.m., the free ammonia figures are at a maximum.

Another possible cause of the rapid increase of free ammonia with the coming of night is that the fixation of nitrogen from ammonia may cease with the disappearance of sunlight.

During the day the photosynthetic reactions of the plants remove CO_2; at night, when photosynthesis ceases, carbon dioxide is still being liberated by plant and animal respiration with the result that a certain amount of normal carbonate is converted to bicarbonate and the p_H value of the water falls to its minimum during this period.

River Lark. Series II.

On April 27/28th, at Icklingham, a further set of observations over a period of 24 hours was made confirming the results obtained on March 30/31st. The figures are given in Table III and graphically in Fig. 2.

Table III.

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
6 p.m.	11	1·532	137	0·02	7·9–8·0	Bright sunshine
7 ,,	10·6	1·463	130	0·02	7·9–8·0	Good light. Sundown
8 ,,	10	1·422	124	0·02	7·9–8·0	Dusk
9 ,,	10	1·291	112	0·025	7·9–8·0	Dark. Fine
10 ,,	9·5	1·177	101	0·02	7·9–8·0	Clear and starry. No moon. Fine
11 ,,	9·5	1·058	91	0·02	7·8	,, ,, ,,
Midnight	9	0·960	82	0·03	7·7–7·8	,, ,, ,,
1 a.m.	8·5	0·808	68	0·09	7·6–7·7	,, ,, ,,
2 ,,	8	0·738	61·5	0·13	7·6–7·7	,, ,, ,,
3 ,,	8	0·702	58·5	0·14	7·6	Sky just lightening. Fine
4 ,,	8	0·710	60	0·16	7·5–7·6	
5 ,,	7	0·708	57	0·15	7·5–7·6	
6 ,,	7	0·723	58	0·15	7·5–7·6	
7 ,,	7	0·792	64	0·15	7·5–7·6	
8 ,,	7·5	0·894	73	0·15	7·6–7·7	Bright sunshine. Fine
9 ,,	7·8	1·005	83	0·14	7·6–7·7	,, ,,
10 ,,	8·5	1·212	102	0·1	7·7	,, ,,
11 ,,	9·5	1·459	126	0·04	7·7–7·8	,, ,,
Midday	9·7	1·580	137	0·03	7·8	,, ,,
1 p.m.	10·5	1·787	158	0·02–0·025	7·9–8·0	,, ,,
2 ,,	10·6	1·889	167	0·02–0·025	8·0	,, ,,
3 ,,	11·2	1·969	176	0·02–0·025	8·0	,, ,,
4 ,,	11·2	1·995	178	0·02	8·0	Cloudy. Fine
5 ,,	11·2	1·863	168	0·02	8·0	,, ,,
6 ,,	11·5	1·807	163	0·02	8·0	,, ,,

Sunset April 27th, 7.15 p.m.
Sunrise ,, 28th, 4.40 a.m.
Sunset ,, 28th, 7.16 p.m.

April 27th was a bright sunny day, following several bright days, whereas on March 30th the morning, although fine, was not so bright and the afternoon was dull. The effect of this difference in the weather conditions is seen in the greater difference between the maximum and minimum values of percentage saturation of oxygen, viz.:

March 30th	Maximum	157 % at 3 p.m.
April 27th	Maximum	178 % at 5 p.m.
March 30th	Minimum	65 % from 1 a.m. to 7 a.m.
April 27th	Minimum	57 % from 4 a.m. to 7 a.m.

The effect of the longer period of light on April 27/28th, compared to that on March 30/31st, is seen in the shorter period of minimum of oxygen during the night. By superimposing the March and April curves these differences are very clearly shown.

RIVER ITCHEN. SERIES I.

For the purposes of comparison a similar experiment was made on the River Itchen in Hampshire. The general topography of this stream has been previously described [Butcher, 1927]. The Itchen is not polluted in any way. The place of sampling was immediately above a small weir at the Ministry of Agriculture and Fisheries' Research Station, Alresford, where the river is about 2 feet deep and 20 feet wide, and its speed of flow is about 1300 yards per hour.

The results are given in Table IV and graphically in Fig. 3.

Table IV.

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 p.m.	9·4	1·050	90·5	<0·01	8·0	Dark
10 ,,	9·0	1·053	90	<0·01	7·8	Clouds obscuring moon
11 ,,	8·6	1·024	87	<0·01	8·0	
Midnight	8·0	1·027	86	<0·01	7·8	Moonset
1 a.m.	8·0	0·979	82	<0·01	7·7	
2 ,,	7·8	0·995	82	<0·01	7·8	
3 ,,	7·5	1·035	85	<0·01	7·6	Raining
4 ,,	7·5	0·978	80·5	<0·01	7·5	,, Sky just lightening
5 ,,	7·3	0·974	80	<0·01	7·6	,, Dawn
6 ,,	7·1	0·985	81	<0·01	7·5	,,
7 ,,	7·1	1·040	85	0·01	7·5	,,
8 ,,	7·1	1·095	90	0·0015	7·5	,, River rising and turbid
9 ,,	7·1	1·135	93	0·02–0·025	7·6	,,
10 ,,	7·0	1·135	93	0·01	7·6	,,
11 ,,	7·0	1·199	97	0·01	7·5	,, slightly
Midday	7·3	1·321	108	0·015	7·4	,, ,,
1 p.m.	7·4	1·322	109	0·01	7·6	,, ,,
2 ,,	7·8	1·349	112	<0·01	7·6	Fine. Dull
3 ,,	8·0	1·247	104	<0·01	7·6	Brighter. No sun, cold wind
4 ,,	8·1	1·280	107	<0·01	7·6	Fine. Storm at 4.30
5 ,,	8·2	1·313	109	<0·01	7·5	Fine
6 ,,	8·0	1·279	107	<0·01	7·6	Sun shining
7 ,,	8·0	1·121	93	<0·01	7·6	Sunset
8 ,,	7·5	1·075	88	<0·01	7·5	Clear night
9 ,,	7·0	0·994	81	<0·01	7·5	,,

Sunset April 6th, 6.39 p.m.
Sunrise ,, 7th, 5.25 a.m.
Sunset ,, 7th, 6.41 p.m.

Variation in dissolved oxygen.

The dissolved oxygen curve is similar to that obtained from the Lark figures. The variation is less, ranging from 0·974 parts per 100,000 to 1·349 parts per 100,000 compared to 0·795 and 1·803 for the Lark.

Variation in ammoniacal nitrogen.

The ammoniacal nitrogen content is normally less than 0·01 parts per 100,000. It seems probable that the higher values of 0·01 to 0·025 obtained between 7 a.m. and 1 p.m. are due to the washing in of ammonia compounds from the soil by flood-water. There is in the Itchen nothing comparable to the diurnal variation of ammonia in the Lark.

Variation in p_H.

The maximum p_H value, 7·8–8·0, was obtained from 9 p.m. until 1 a.m. after which a rapid decrease was observed to 7·5–7·6 at 3 a.m., the reaction remaining constant at this figure for the remainder of the period of observation.

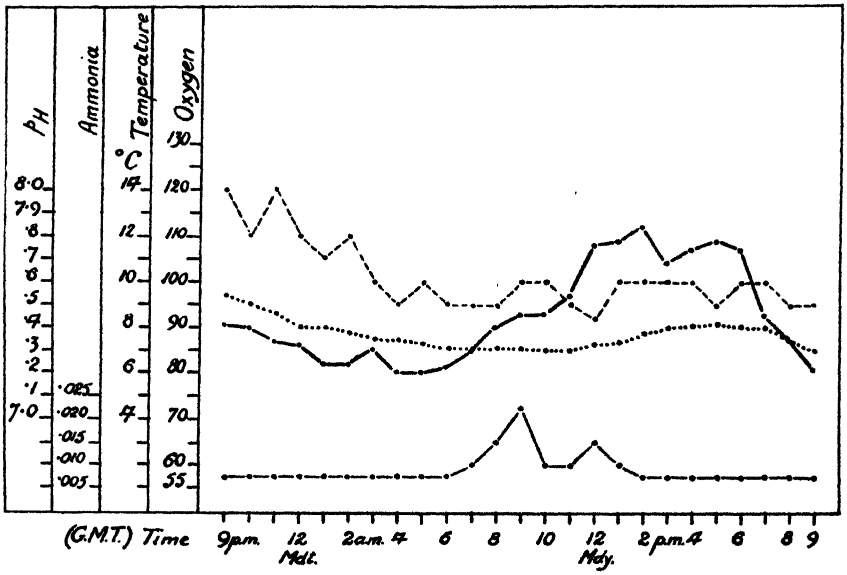

Fig. 3. River Itchen. April 6/7th, 1927.

——————— Oxygen, percentage saturation of dissolved

-·-·-·-·- Ammoniacal nitrogen, parts per 100,000

— — — — — p_H value .... Temperature °C

Variation in temperature.

The temperature curve showed a minimum from 6 a.m. to 11 a.m., after which it increased steadily to a maximum at 5 p.m.

RIVER ITCHEN. SERIES II.

A further set of observations was made on the Itchen on May 4/5th and the results are given in Table V and graphically in Fig. 4.

Table V.

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	p_H value	Remarks
6 p.m.	11·6	1·170	105	8·0	Cloudy. Fine
7 „	11·3	1·043	93	7·8	Dull. Raining
8 „	11·0	0·974	87	7·7	Dusk. Dull. Raining
9 „	11·0	1·000	89	7·6	Dark. Raining
10 „	10·8	0·935	83	7·6	Cloudy. Fine
11 „	10·6	0·939	83	7·55	„ „
Midnight	10·3	0·897	79	7·55	„ „
1 a.m.	10·1	0·886	77	7·6	„ „
2 „	10·0	0·885	77	7·55	„ „
3 „	10·0	0·882	77	7·55	Sky just lightening. Cloudy. Fine
4 „	10·0	0·875	76	7·55	Dawn. Cloudy. Fine
5 „	10·0	0·893	78	7·5	Sunrise. Cloudy
6 „	9·9	0·949	83	7·55	Bright
7 „	9·9	1·035	90	7·5	Cloudy. Fine
8 „	10·0	1·135	99	7·6	„ „
9 „	10·1	1·253	109	7·55	„ „
10 „	10·4	1·170	103	7·7	Heavy thunderstorm at 9.45
11 „	10·2	1·170	102	7·6	Very dull. Raining
Midday	10·3	1·214	106·5	7·6	Raining
1 p.m.	11·2	1·285	115	7·75	Sun shining
2 „	12·8	1·409	130·5	8·0	„
3 „	13·2	1·302	122	8·0	„
4 „	13·7	1·299	122	7·9	„
5 „	13·8	1·325	124	7·9	„
6 „	13·6	1·255	118	7·9	„

Sunset May 4th, 7.26 p.m.
Sunrise „ 5th, 4.27 a.m.
Sunset „ 5th, 7.27 p.m.

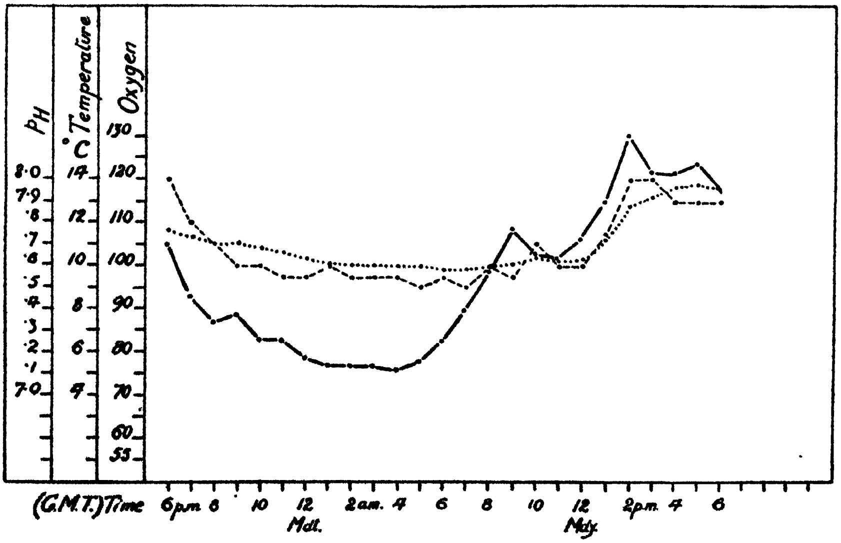

Fig. 4. River Itchen. May 4/5th, 1927.

———————— Oxygen, percentage saturation of dissolved

— — — — — — — p_H value .... **Temperature °C.**

Variation in dissolved oxygen.

This curve shows greater similarity to those obtained during the Lark experiments. The greater difference between the maximum and minimum values, 1·409 and 0·875 parts per 100,000 respectively, is due to the brighter day of May 5th compared with April 7th. The irregularity in the oxygen curve after 9 a.m. was coincident with a heavy thunderstorm, the effect of which is also shown on the p_H and temperature curves.

Variation in p_H.

The p_H dropped gradually from 8·0 at 6 p.m. to 7·5 at 11 p.m., at which value it remained constant until 9 a.m. when it began to rise, was checked by the storm, and then went on to 8·0 at 3 p.m. After 4 p.m. a slight fall began.

Variation in temperature.

The temperature shows a regular fall from 11·6° at 6 p.m. to 9·9° at 7 a.m., after which a regular rise, again checked by the storm, to 13·8° occurred.

Variation in ammonia.

As in the experiment of April 6/7th, the ammoniacal nitrogen content showed no significant variation comparable to that obtained on the River Lark.

DISCUSSION OF RESULTS.

Dissolved oxygen curves.

All four curves show a similarity of structure with a minimum during darkness and a maximum 2 or 3 hours after noon.

Owing to the continuous absorption of oxygen by all organisms during the night, it seems that the oxygen content should fall continuously to a minimum value at dawn. In practice each curve shows a minimum *period* extending over several hours of the night, and it seems probable that an equilibrium is established between solution of oxygen from air and absorption of oxygen by organisms. The time-lag after dawn and after noon is in accordance with the known facts of photosynthesis [Stiles, 1925].

It will be noticed that the difference between the maximum and minimum values of oxygen obtained in the River Lark is considerably greater than that in the case of the Itchen and a comparison of the physical conditions of the two rivers is made below.

River Lark	*River Itchen*
Speed of flow 600 yards per hour	Speed of flow 1300 yards per hour
Depth 4–5 ft.	Depth 2–3 ft.
Polluted during the winter by effluents from beet-sugar factory	Unpolluted
Bottom—mud containing decomposing organic matter	Bottom—clean stones
Higher green plants very small and young: *Sparganium simplex* and *Potamogeton lucens* dominant	Higher green plants in some quantity and of continuous growth: *Callitriche stagnalis* and *Ranunculus penicillatus* dominant

Since the River Itchen is the more rapid and shallow stream, it would be expected that the greater agitation would keep the oxygen content closer to the saturation value than in the slower and deeper River Lark.

The spring maximum of diatoms in the River Lark is considerably greater than in the River Itchen (possibly due to the recent pollution). This difference in the quantity of diatoms causes increased photosynthetic production of oxygen resulting in the higher values of supersaturation obtained on the River Lark.

The oxidation of the organic matter in the mud of the River Lark would tend to give lower minimum oxygen values than in the Itchen, where mud is absent, and this is borne out by the minimum values obtained.

The larger quantity of higher green plants in the Itchen would tend to increase the maximum oxygen values, but, at the time of the experiment, diatom activity overshadows that of the larger plants.

SUMMARY.

Day	*Night*
Photosynthesis by plants causes assimilation of carbon dioxide and liberation of oxygen, while decay of organic matter produces ammonia which is removed by the high concentration of oxygen, resulting in:	Photosynthetic production of oxygen ceases, and the ammonia produced by organic decay is not removed. Animal and plant respiration continues, taking up oxygen and producing carbon dioxide, resulting in:
Oxygen maximum.	*Oxygen minimum.*
Ammoniacal nitrogen minimum.	*Ammoniacal nitrogen maximum.*
p_H value maximum.	*p_H value minimum.*

In conclusion, the authors wish to express their thanks to the Ministry of Agriculture and Fisheries and to Sir R. Robertson, the Government Chemist, for permission to publish these results and to Mr A. C. Gardiner, of the Ministry of Agriculture and Fisheries for his assistance in the Itchen experiment.

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Note added August 16th. Since writing the above we have seen a reference to a paper by B. Seydel "Ueber die Schwankungen des Sauerstoffgehaltes in Teichen" (*Mitt. d. Fisch-Ver. f. d. Prov. Brandenburg.* Bd. 4, 1912–13) in which are given figures for the diurnal variation of the oxygen content of ponds. His work seems merely to amplify that of Morren [1841].

CXXVIII. BLOOD-FAT AND EXERCISE.

By JAMES WATERS THORNTON PATTERSON.

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(*Received June 19th, 1927.*)

In the course of a series of investigations into the variations of the fasting blood-fat level on diets of low and high fat contents a very definite increase after exercise was noticed. This was accidentally discovered in samples withdrawn after a game of hockey, and was quite distinct from the original line of research, but so definite as to warrant a separate investigation.

EXPERIMENTAL.

The method of estimation of the blood-fat content followed in this paper is that of Stewart and White [1925] and has been found eminently satisfactory.

In spite of the lengthy series of manipulations involved it was found possible to estimate the fat in a solution of known concentration within 3 %, while duplicate samples of blood gave results differing by no more than 2 %.

In addition to the warnings given by Stewart and White, the author has found it necessary to redistil all the anhydrous spirit used with sodium hydroxide, as the slight acidity of the spirit supplied was sufficient to interfere with the accuracy of results. He has, furthermore, found it necessary to interpose a CO_2 trap between the siphon system and the capillary distributing the air current to the titrating fluids. This trap consists of a concentrated solution of sodium hydroxide through which the air bubbles, having passed through the siphon system. To rely entirely on a soda-lime tower guarding the inlet of the siphon system was unsatisfactory, and, moreover, was not effective when interchange of siphon bottles had to take place. By placing this trap beyond the siphon system, however, one can interchange the bottles without any fear of the air supply becoming contaminated with CO_2.

The blood-sugar was estimated by Benedict's method [1918]. The results are all high and in later investigations the method of Folin and Wu [1920] has been used. In a series of estimations comparing the results of estimation by both these methods, the Benedict technique has been observed to give results consistently 20 % above those by the Folin-Wu method.

The cholesterol content was determined by Bloor's modification [1915] of the Autenrieth-Funk process [1913].

The air samples for the determination of the respiratory quotient were collected by means of the Douglas Bag [1911] with subsequent analysis by means of Haldane's [1918] gas analysis apparatus.

The percentage of the corpuscles was estimated by the haematocrit.

RESULTS.

Preliminary experiments (Table I) showed that the fasting fat content remained remarkably constant for a given individual over a considerable period of time.

Table I.

Subject	Date	Fasting level mg. per 100 cc.	% variation
E. M. F.	Nov. 17, 1926 Feb. 16, 1927	661 615	6·5
I. H. M.	Dec. 4, 1926 Feb. 28, 1927	535 520	3·0
A. J.	Nov. 27, 1926 Mar. 2, 1927	696 690	1·0
W. M. A.	Dec. 3, 1926 Feb. 14, 1927	612 627	3·0
M. C. M.	Nov. 12, 1926 Feb. 10, 1927	439 433	1·5

The results of the effect of exercise are shown in Table II.

In each individual case the same amount of exercise has been performed (1) in the fasting state, (2) after the administration of 100 g. glucose by the mouth. In all cases specimen *A* is a basal fasting sample. In the experiments where glucose has been given the 100 g. are taken immediately after the collection of specimen *A*, and specimens *B* and *C* are collected at intervals of ½ and 1 hour respectively, the patient resting in the interval. Specimen *D* is in all cases the sample immediately after exercise. Specimens *E* and *F* are collected at the times noted with the patient resting after exercise.

The subjects of the experiments were all healthy medical students.

Case E. M. F. Nov. 17, 1926.

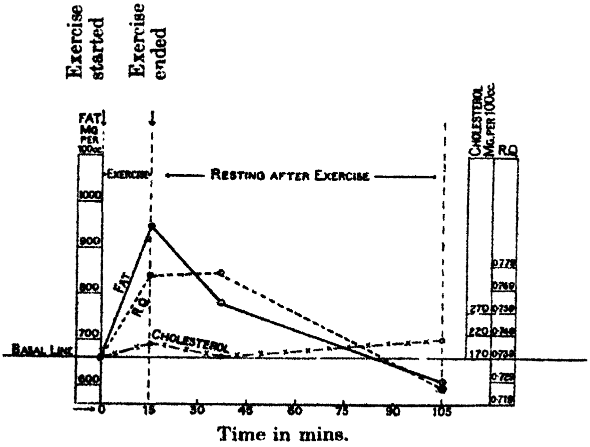

Fig. 1. Showing effect of exercise in fasting individual.

Figs. 1 and 2 represent in graphic form each type of change.

Fig. 1 (case E. M. F.) shows the effect of exercise in the fasting individual. Cholesterol and R.Q. changes are recorded.

Table II.

Case	Specimen	Remarks	Time	Fat (mg. per 100 cc.)	Sugar (mg. per 100 cc.)	Cholesterol (mg. per 100 cc.)	% corpuscles in blood	R.Q.	Work done
(1)* E. M. F.	*A*	Fasting	9.35 a.m.	661·5	—	169·4	—	0·739	Running along definite route for 15 minutes
	D	After exercise	9.55 „	947·2	—	202·0	—	0·775	
	E	Resting	10.15 „	779·9	—	173·9	—	0·776	
	F	Resting	11.45 „	609·7	—	212·7	—	0·726	
(2)†	*A*	Fasting	10.10 a.m.	615·6	119·0	—	38	—	Running along same route for same time
	B	½ hour after sugar	10.40 „	627·5	154·0	—	—	—	
	C	1 hour after sugar	11.10 „	577·2	129·0	—	32	—	
	D	After exercise	11.30 „	571·2	285·0	—	52	—	
	E	Resting	12.5 p.m.	461·7	222·0	—	—	—	
(1)* I. H. M.	*A*	Fasting	10.30 a.m.	520·9	160·0	—	34	—	Bicycle ergometer. Work done = 28,632 kg. metres
	D	After exercise	11.0 „	701·5	270·0	—	38	—	
	E	Resting	11.5 „	734·0	116·0	—	57	—	
	F	Resting	11.30 „	686·7	163·0	—	46	—	
(2)†	*A*	Fasting	10.15 a.m.	535·7	—	200·0	—	—	Bicycle ergometer. Repetition of above exercise. Work done = 28,632 kg. metres
	B	½ hour after sugar	—	—	—	—	—	—	
	C	1 hour after sugar	11.15 „	515·0	—	215·0	—	—	
	D	After exercise	11.40 „	467·6	—	217·0	—	—	
	E	Resting	12.20 p.m.	518·0	—	208·0	—	—	
(1)* A. J.	*A*	Fasting	10.45 a.m.	695·6	138·0	—	40	—	Bicycle ergometer. Work done = 37,794 kg. metres
	D	After exercise	11.0 „	843·6	235·0	—	39·6	—	
	E	Resting	11.25 „	858·4	253·0	—	40	—	
	F	Resting	11.35 „	666·0	202·0	—	38	—	
(2)†	*A*	Fasting	10.10 a.m.	689·6	126·0	208·3	—	—	Bicycle ergometer. Repetition of above exercise. Work done = 37,794 kg. metres
	B	½ hour after sugar	—	—	—	—	—	—	
	C	1 hour after sugar	11.10 „	680·8	235·0	204·0	—	—	
	D	After exercise	11.25 „	710·4	135·0	204·0	—	—	
	E	Resting	12.15 „	666·0	154·0	212·7	—	—	
(1)* W. M. A.	*A*	Fasting	10.18 a.m.	627·5	160·0	—	—	—	Bicycle ergometer. Work done = 57,266 kg. metres
	D	After exercise	10.40 „	864·3	227·0	—	—	—	
	E	Resting	11.10 „	488·4	175·4	—	—	—	
	F	Resting	11.30 „	370·0	196·0	—	—	—	
(2)†	*A*	Fasting	9.50 a.m.	612·7	—	198·0	—	—	Bicycle ergometer. Repetition of above exercise. Work done = 57,266 kg. metres
	B	½ hour after sugar	—	—	—	—	—	—	
	C	1 hour after sugar	10.50 „	603·8	—	190·4	—	—	
	D	After exercise	11.20 „	580·6	—	202·0	—	—	
	E	Resting	—	—	—	—	—	—	
(1)* M. C. M.	*A*	Fasting	9.20 a.m.	439·5	—	210·4	—	0·763	Running along definite route for 15 minutes
	D	After exercise	9.35 „	597·9	—	197·2	—	0·798	
	E	Resting	10.5 „	630·4	—	206·9	—	0·787	
	F	Resting	10.40 „	598·8	—	200·0	—	—	
(2)†	*A*	Fasting	10.13 a.m.	433·6	120·0	—	53	—	Running along same route for same time
	B	½ hour after sugar	10.43 „	245·6	206·2	—	46	—	
	C	1 hour after sugar	11.13 „	553·5	176·9	—	54	—	
	D	After exercise	11.32 „	429·2	281·7	—	—	—	
	E	Resting	11.57 „	432·1	217·3	—	42	—	

* Without glucose. † With glucose.

Fig. 2 (case M. C. M.) shows the effect of exercise after the ingestion of 100 g. of glucose. The relation of the sugar and fat curves during the first hour of glucose absorption (the subject resting during this period) is discussed later.

DISCUSSION.

The most striking feature of these experiments is the constant increase in the blood-fat content after exercise in the fasting individual, an increase which does not occur after the ingestion of glucose (Figs. 1 and 2).

Case M. C. M. Feb. 10, 1927.

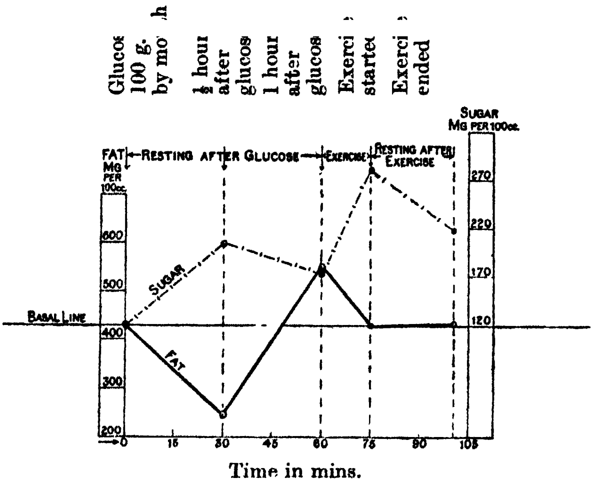

Fig. 2. Showing effect of exercise after glucose (100 g).

The increase obtained is well beyond the margin of experimental error. Thus in case E. M. F. the blood-fat content is increased by 43 %, in I. H. M. by 35 %, in A. J. by 21 %, in W. M. A. by 37 %, and in M. C. M. by 36 %.

It may be argued that this increase is due entirely to the concentration of the blood consequent upon the severe exercise, but in cases E. M. F. and M. C. M., where this question was investigated, the increase in concentration of the blood corpuscles was found to be 4 % in the former and nil in the latter, while the increase in fat content amounted to 35 % and 21 % respectively. Similar findings have resulted from the investigation of this point in cases not yet published, so that one is in a position to state that the registered increase of blood-fat after exercise is greater than that which would result from simple concentration of the blood after exercise and is, in fact, a real and not an apparent increase.

Having established the fact of a definite increase in blood-fat after exercise, one or two other points require to be settled before discussing its significance.

In the first place Leathes and Raper [1925] do not regard the variations of blood-fat content in samples of blood taken "from any accessible vein" as being of any value whatsoever in determining the nature and mechanism of fat transport and utilisation. The significance of such data derived from examination of the blood is discussed in a paper by Lyon [1923] dealing with some factors which influence dosage. Secondly, before a variation in blood-fat content can be of any importance a constant must be established. If we have no constant the increase in blood-fat after exercise loses much of its significance, since such increase might be due to normal fluctuation of the blood-fat level.

The constancy of the blood-fat content in fasting individuals.

Most observers hitherto have found extreme variations in the blood-fat fasting content in successive examinations and White [1926] has mentioned the possible factors influencing the blood-fat level and "the difficulty of obtaining any definite correlation between them."

Terroine, on the other hand [1914], working with dogs, found that any one dog would show a similar amount of fat in the blood provided the conditions remained similar (the amount being subject to a variation of 15 % either way). He found considerable variation, however, in comparing the fat contents of different dogs under similar conditions. In other words, subject to a variation of 15 %, the individual fasting fat content remained steady under similar conditions. This latter view is borne out by the results of this series of experiments on men, save that here the percentage variation is much less. Thus it will be noted (Table I) that while the range of fasting blood-fat content in the five cases of normal students is from 439 mg. to 695 mg. per 100 cc., the fasting blood-fat level in the individual shows a definite tendency to remain constant, and that the increase of blood-fat after exercise is a real and not an apparent increase.

From Table II it is evident that a very definite increase in the blood-fat content occurs after exercise in the fasting individual. It is realised that the exercise involved in a patient moving from one room to another is sufficient to upset the estimations of basal metabolism and blood-pressure for an hour afterwards. It would appear, then, that the variations in fasting blood-fat content found hitherto by other observers may be accounted for by the fact that insufficient attention had been paid to the importance of the effect of exercise as a disturbing factor.

No basal sample was withdrawn in any of the present series of cases until the patient had rested on a couch for an hour.

The increase of the blood-fat content after exercise.

This increase may be due to either of two factors. It may be a definite response to a demand for more fat on the part of the working cells, or it may be due to a non-removal of the fat from the blood-stream, the working cells either refusing it or being unable to utilise it.

The first hypothesis appears to be the more logical.

This increase in blood-fat content after exercise has been remarked upon by Gage and Fish [1924], who determined the number of fat particles in the blood by microscopic dark-field, high-power investigation. While the method seems somewhat crude, these observers noted a definite increase in fat particles, or "chylomicrons" as they call them, after exercise, and they interpreted the results as meaning that in the ordinary activity of a fasting man the fat is poured out into the blood from the depôts only as it is needed and at the rate it is needed.

They found that it took some time for the fat content to resume normal proportions, and liken the effect of exercise to that of absorption after a fatty meal where the return of the fat to its previous level is slow, the fall being due to "metabolism or assimilation or both as in the curve of digestion."

While this point of the slow return of the blood-fat content to fasting level was not investigated in this series of experiments, the indications are not in support of this finding, for in no case was the examination maintained beyond 2 hours after the exercise, and yet in three of the five cases the blood-fat content had already returned to, or gone lower than, its previous fasting level.

The variations of the blood-fat content after exercise when 100 *g. glucose had been taken prior to the exertion.*

The increase in fat content does not take place if the patient has had glucose before the exercise, despite the fact that the amount and the severity of the exercise were the same in both the investigations with glucose and without it. In all cases the fat levels after exercise when glucose has been previously taken remain practically constant (Fig. 2).

This suggests that the working cells only call upon the fat when they have insufficient carbohydrate readily available.

The respiratory quotient in relation to exercise.

The existing views on this question are somewhat conflicting and not only do the interpretations of experimental data differ, but the actual results of similar experiments vary greatly.

Some observers [see Chauveau, 1896] maintain that carbohydrate alone furnishes the energy of muscular work and that fat can only be utilised by first undergoing conversion into carbohydrate.

Others [Zuntz, 1896; Loewy, 1908; Katzenstein, 1891] find no difference in R.Q. before and during muscular work, or [Benedict and Cathcart, 1914] maintain that this is true only for light work, and that during heavy muscular work the R.Q. is slightly raised.

Another theory [Atwater and Benedict, 1903] is that the working cells utilise fat and carbohydrate in the proportions in which they are presented to them, and that if fat is to be used in the production of muscular energy, then it is oxidised directly and does not undergo a preliminary conversion into sugar.

More recently Krogh and Lindhard [1920, 1, 2] suggest that during rest and during work the proportion of fat and carbohydrate catabolised is a function of the available supplies of these substances, and that carbohydrate is formed from fat and stored when the R.Q. is below 0·8, while a corresponding transformation of carbohydrate to fat takes place when the R.Q. is above 0·9.

Benedict and Cathcart [1914] find that the R.Q. rises during prolonged and heavy work, but when the work is finished the R.Q. falls below that observed previous to the performance of the work.

The investigations into this question in the present series of cases are not sufficiently extensive to warrant any sweeping deductions. In the two cases in which the R.Q. was followed up (cases E. M. F. and M. C. M.) it will be noticed (*a*) that the initial basal R.Q. is low, (*b*) that immediately after exercise the quotient tends to rise slightly, and (*c*) after an interval of rest the R.Q. has fallen again (in one case (Fig. 1) it has fallen below that observed previous to the performance of the exercise).

Taking into account the noted increase in blood-fat after exercise, one might interpret these changes as follows.

(i) That, on the basis of the observations of Krogh and Lindhard, in the initial fasting period the low R.Q. suggests that fat is being converted into carbohydrate and stored.

(ii) That during exercise the stored carbohydrate is utilised, thus tending to force the quotient up towards unity, which is the R.Q. for pure carbohydrate combustion. Coincidently, more fat is poured out for conversion into sugar to maintain the supply of carbohydrate, so tending to lower the R.Q. The resultant slight increase in R.Q. noted immediately after exercise is the mean of these two effects.

(NOTE. The R.Q. was not taken during exercise, but immediately after it, when, it may be assumed, the physiological mechanism at work during exercise had not had time to readjust itself to resting conditions.)

(iii) After exercise, during the resting period, the depleted carbohydrate store is restored by the transformation of fat into carbohydrate with the consequent fall in R.Q. which such interconversion entails.

The relationship between the blood-sugar and blood-fat contents.

Although our knowledge at present remains insufficient for any very definite conclusions to be reached, there are certain indications that the blood-sugar and blood-fat contents are in some way related.

The evidence for such a relationship adduced in the present investigations may be briefly summarised as follows.

(1) In case M. C. M. it is noticed that after the taking of glucose (the individual resting), while the blood-sugar rises in the first half-hour and falls in the next half-hour, the blood-fat content does exactly the reverse. This has proved to be a fairly constant factor in many cases not yet published (Fig. 2).

(2) This apparent relationship in the resting individual between the blood-sugar and blood-fat, where, during absorption of sugar, the peak of the blood-sugar curve coincides with the trough of the blood-fat curve, is not maintained during exercise.

Here all the cases of exercise without glucose show an increase of blood-sugar side by side with an increase of blood-fat immediately after exercise, while in those cases where glucose was given before the exercise was started we have no increase in blood-fat but definite rise of blood-sugar in two cases and a definite fall in another case. These changes are quite definite even when allowance of 10 % error is made for the colorimetric method of estimating the blood-sugar content.

The relation of cholesterol with the variations of blood-fat content.

Terroine [1914] found that the ratio of fatty acid to cholesterol was almost constant during the absorption of fat.

This was not substantiated by Bloor [1915], who found the variations of cholesterol during fat absorption to be irregular, small, and sometimes barely appreciable. Other observers [Hiller, Linder, Lundsgaard and Van Slyke, 1924] have confirmed Bloor's observation.

The present conception of the behaviour of cholesterol during the absorption of fat, stated briefly, is that up to a certain point the cholesterol tends to increase, but not beyond this level, depending, perhaps, on the amount available in the intestine from food or bile. The association of cholesterol with fat does not appear to be essential either to the process of absorption or of transfer from blood to tissues.

In the present series of cases where the cholesterol content was estimated it is necessary to modify the figures on account of the colorimetric method of estimation. This means an error either way of 10 %, and worked on this basis it will become evident that there is nothing conclusive in the variations—indeed, it may be deduced that the cholesterol content is unaffected, although it would be unwise to state definitely that no change occurs.

SUMMARY.

1. Figures are presented indicating a remarkable constancy of the fasting blood-fat content for the same individual.

2. A definite increase in the blood-fat content is noted as occurring in fasting individuals immediately after severe exercise.

3. There is no increase in blood-fat after exercise in the same individuals when they have received 100 g. glucose *per os* prior to the muscular exertion.

4. The variations of the R.Q. are discussed and the suggestion made that, taken in conjunction with the observed increase in blood-fat in fasting individuals after exercise, they might indicate that fat was being mobilised for its conversion into carbohydrate.

5. Indications of a relationship between the blood-fat and blood-sugar contents are noted.

6. The investigation of the cholesterol content of the blood seems to indicate that during the period of observation it remained unaffected by the exercise and, apparently, unrelated to the definite blood-fat variations.

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CXXIX. THE DISPERSED PHASE OF THE BLOOD CORPUSCLES.

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(*Received November 3rd, 1926.*)

A. Gough in an extremely interesting paper [1924] on "The nature of the red blood corpuscle" also treats the question of the dispersed phase of the blood corpuscle. He determines the dispersed phase by computing the changes of volume of the blood corpuscles in fluids of different osmotic pressure by Hamburger's principle [1898], and he comes to the conclusion that the dispersed phase constitutes two-thirds of the volume of the blood corpuscle, while the watery phase, in which the electrolytes, etc., are found to be dissolved, only forms one-third of the blood corpuscle.

The value which Gough indicates for the dispersed phase of the blood corpuscle is even greater than that which Hamburger has found, but which, according to my investigations, must be said to be too high.

It need not be specially emphasised that the knowledge of the volume of the dispersed phase is of great interest, for not till the magnitude of the dispersed phase in the plasma and blood corpuscles be known will it be possible to discuss and explain the empirically found relations of partition which have been determined for non-electrolytes capable of permeating the membrane of the blood corpuscle. Thus, if one finds that the partition of urea and acetone between the blood corpuscles and the plasma corresponds to the proportion between the magnitude of the dispersion medium in the two systems, the most reasonable explanation will be that the two substances are only found dissolved in the dispersion medium of the two systems. On finding, however, that

$$\frac{\text{Concentration in the blood corpuscles}}{\text{Concentration in the plasma}} \gtrless \frac{\text{Dispersion medium in the blood corpuscles}}{\text{Dispersion medium in the plasma}}$$

after equilibrium has been established, it is seen that the partition cannot simply be interpreted by referring to the difference of volume of the dispersion medium. The substance in question must also be soluble in the dispersed phase of the one system, being adsorbed to the same or complexly bound.

It is therefore of great importance for the comprehension of these relations to ascertain whether the value for the dispersed phase found by Gough [1924], or that found by Ege [1920], is correct.

Gough's findings are:

Dispersion medium 30–35 % Dispersed phase 70–65 %.

Ege finds:

Dispersion medium about 60 % Dispersed phase 40 %.

By determinations performed by Hamburger's principle, I came to the result that the changes of volume of the blood corpuscles in a cane-sugar solution varied in the manner which would be expected in accordance with osmotic theories, if the dispersed phase of the blood corpuscles was 35–45 % [Ege, 1922].

When Gough, using the same method, finds a value about twice as great, this may be partly due to the circumstance that the volume of the blood corpuscles does not keep constant in such NaCl solutions as he used, but particularly to an incomplete sedimentation of the column of blood corpuscles in those experiments where the blood corpuscles are suspended in a hypertonic salt solution, which renders sedimentation much more difficult than iso- and hypo-tonic solutions [Ege, 1923]. Gough only says that all the samples were spun in a water-centrifuge for 3 hours.

Gough does not think that this has led to a complete sedimentation of the blood corpuscles, but he considers the error which will arise from this as negligible. However, he has furnished no proof of this being actually the case, and, as will easily be seen, an incomplete sedimentation may bring about a very considerable error in his determination and computation of the volume of the dispersed phase.

If the centrifuging of the blood corpuscles is continued until the whole of the column of blood corpuscles acquires the appearance of laked blood, that is to say, until an absolute sedimentation is attained, one will find that different periods of centrifuging will be required, according as the blood corpuscles are suspended in hypo- and iso-tonic solutions in which the absolute sedimentation takes place relatively rapidly, or in hypertonic solutions, with which a prolonged centrifuging is required if the same quantitative sedimentation is to be obtained. If all the samples are centrifuged for an equally long time, the values found for the volume of the blood corpuscles may become too high, particularly in the hypertonic solutions.

Even fairly small errors may lead to very great errors on computation of the volume of the dispersed phase. Thus, supposing that a complete sedimentation is attained in experiments with an isotonic fluid as well as with a fluid whose osmotic concentration is 50 % higher, the dispersed volume will be found to be = 40 %, if the first volume be = 100 and the other = 80, as becomes evident from the following formula:

$$1{\cdot}00\,(100 - x) = 1{\cdot}50\,(80 - x),$$
$$x = 40.$$

If, however, on account of imperfect centrifugalisation, the complete sedimentation were attained in the isotonic solution only, but not in the hypertonic solution, so that there still remained, for instance, 12–13 % of fluid among the blood corpuscles, that is to say, that the volume of blood corpuscles amounted to 90 instead of the true volume of 80; the dispersed volume would then be found to be 70 %:

$$1{\cdot}00\,(100 - x) = 1{\cdot}50\,(90 - x),$$
$$x = 70.$$

The method of determining the dispersed phase introduced by Hamburger—employed by Gough—will thus easily lead to excessively high values for the dispersed phase unless the centrifuging is guaranteed to be absolutely quantitative in all the samples.

If the centrifuging is continued until the column of blood corpuscles has acquired its true volume without interstitial plasma, this manifesting itself by the column of blood taking the appearance of laked blood [Köppe, 1905] in its whole extent, a diminution of volume in the hypertonic solutions approximately corresponding to a dispersed phase of 35–45 % will be found, as has previously been mentioned.

As it might be presumed that the envelope of the blood corpuscles will resist the changes of volume, the method mentioned may, however, give rise to some objections. Another determination of the volume of the dispersed phase is derived from making solutions of non-electrolyte in equal volumes of water and blood corpuscle press-juice, and afterwards, determining the change in the freezing-point of the fluid.

8 g. of cane-sugar in water, $\Delta = 1{\cdot}22°$.

8 g. of cane-sugar in the same volume of blood corpuscle press-juice[1], $\Delta = 1{\cdot}81°$.

The volume x of the dispersed phase can then be calculated by the following formula:

$$(100 - x) \times 1{\cdot}81 = 100 \times 1{\cdot}22,$$
$$x = 33\,\%.$$

For the sake of comparison the following determination of dry matter in the blood corpuscle is mentioned:

	Dry matter	Water
Percentage of weight	34	66
Percentage of volume	28	72
	Dispersed phase	Dispersion medium
	33	67

The volume of the dispersed phase is thus 10–15 % greater than the volume found by the determination of dry matter (corresponding however rather closely to the percentage of weight).

SUMMARY

By means of various methods of determination of the volume of the dispersed phase of the blood corpuscle, this volume is found to approach very nearly to the percentage of dry matter of the corpuscle.

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[1] From this value the depression of freezing point of the ordinary corpuscle press-juice has been subtracted.

The figures given by Gough, according to which the volume of the dispersed phase is double the content of dry matter, must be due to a systematic error.

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CXXX. THE INFLUENCE OF VARIATIONS IN THE SODIUM-POTASSIUM RATIO ON THE NITROGEN AND MINERAL METABOLISM OF THE GROWING PIG. II.

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(*Received June 23rd, 1927.*)

Recent investigations on the mineral requirements of the growing animal make it increasingly evident that the adjustment of the proportions of the inorganic constituents of a ration requires as much consideration as the absolute amounts of these elements in the diet. Even though a mineral is present in a ration in sufficient amount for the animal's requirements, the presence of other minerals in unsuitable proportions may lead to insufficient assimilation and retention. The importance in animal nutrition of this balancing of the ration has been frequently emphasised by Orr [1924], who quotes various instances of the interdependence of the different mineral elements. Of special interest in this connection are sodium and potassium. The importance of the ratio of these to each other in the diet was discussed by Bunge as long ago as 1873, and since then the ratio has received more attention than the absolute requirements of each. As a matter of fact, in compounding diets the amounts of these are seldom considered. It is assumed, probably rightly, that any ordinary ration will contain sufficient.

Osborne and Mendel [1918], who carried out a series of growth experiments on rats, drew attention to the surprisingly small amounts of some of the minerals which were sufficient to permit growth. Thus less than 0·04 % of Na in the ration sufficed for normal growth, and with only 0·03 % K considerable growth was likewise attained, although the simultaneous reduction of Na and K to these minimum amounts resulted in nutritive failure. Similarly St John [1923] has found that 0·03 % of K in a wheat ration seemed to furnish an approximate maintenance supply. On the other hand, it seems probable that while these small amounts may be sufficient to cover the minimum requirements of the animal, larger amounts (especially of Na) may be necessary for the purposes of optimum growth and well-being. Thus Olson and St John [1925] have found by varying the amounts of Na in a wheat ration that the most satisfactory results, when both growth and reproduction were considered, were obtained with 0·53 % of Na.

THE Na : K RATIO OF THE RATION.

Though the absolute amounts required are so small, the ratio is of practical importance. The high proportion of K as compared with Na in the most commonly used feeding-stuffs introduces two possible dangers, viz. (1) a Na deficiency may arise not because the small Na requirement of the animal is insufficiently covered by the ration, but because the excessive proportion of K prevents the full utilisation by the animal of the Na actually present, or (2) the excessive proportion of K to Na may cause insufficient assimilation and retention of other elements.

(1) *Effect on* NaCl *content of organism. Bunge's theory.*

The whole question of the sodium-potassium ratio is intimately bound up with the theory of Bunge [1873], which for over 50 years has dominated any reference to the influence of Na and K in nutrition. Bunge, on the ground of very limited experimental data, maintained that excessive intake of potassium salts impoverishes the organism in sodium chloride. He found, in one-day experiments on himself, in which he investigated urinary excretions only, that large doses of K salts caused a large increase in the excretion of Na and Cl, this excessive loss of Na and Cl being however counterbalanced by abnormally low excretion of these elements on the three days following the K intake. Conversely, he found that a dose of sodium citrate caused increased urinary excretion of K with a slight diminution in Cl excretion. Although Bunge himself recognised the incompleteness of his experimental work, pointing out the necessity both for analysing intestinal excretions and for ascertaining the effect of continued high intake of K, he proceeded on this slender foundation to build up and elaborate the theory that has held its ground for half a century. The tendency during this time has been to overlook entirely the limitations of Bunge's experiments, and to regard as a fully demonstrated fact the theory of sodium chloride impoverishment brought about by excess of K salts. Apart from the fact that his data were insufficient to justify the theory even with regard to adult human beings, on whom the experiments were carried out, the hypothesis has been applied indiscriminately both by Bunge and others, and has been generally accepted as true for animals of any species and any age, regardless of the fact that no experimental data whatever were brought forward by him to justify the application of the theory to the young growing animal.

(*a*) *Confirmatory evidence.* Occasionally isolated results have been recorded which appear to support his theory, *e.g.* Luithlen [1912], in feeding a rabbit on green food, obtained a negative balance for Na, along with a retention of Ca, Mg and K. This he regards as an explanation of the herbivores' necessity for sodium chloride, and as a support for Bunge's theory, but the data he presents are too meagre to justify any such general conclusion. Similarly Gérard [1912], working on dogs and mice, believed that he had obtained evidence in support of the theory of the de-sodifying action due to K salts. But the balance experiments reported by him are very short and inconclusive, while his analysis of the organs of the animals is equally inconclusive, in the absence of reliable figures for the normal content of the organs in Na and K. Again, Blum, Aubel and Hausknecht [1921], working on a nephritic patient, found that administration of K salts on a Na-poor diet caused increased excretion of Na, coupled with diuresis. The converse part of Bunge's theory, viz. increased K elimination by intake of Na salts, has also received occasional confirmation, *e.g.* Oehme [1925] states that addition of sodium chloride or sodium bicarbonate to the diet increases the elimination of K, but no details are given in the paper available. Hagentoren and Kozerski, quoted by Shohl [1923], found that sodium citrate (and sodium carbonate) increased the urinary output of K and Cl, but the excessive excretion gradually diminished. Meyer and Cohn [1911], who carried out balance experiments on infants, found that sodium chloride increased the urinary elimination of Cl, Na and K, the K balance being negative. There was a gain in weight which they attributed to a retention of Na and associated water. They found that K salts as a rule, especially the bicarbonate, caused loss of weight and increased elimination of water, Na, K and Cl. These results are quoted from Whelan [1925], the original paper not being available.

(*b*) *Contradictory evidence.* A few results have also been recorded which are not in agreement with the theory, *e.g.* Harnack and Kleine [1899] found that K salts of vegetable acids increased urinary Cl only to a very insignificant extent. Similarly v. Hoesslin [1910], who carried out metabolic experiments on dogs, considered that Bunge's theory was not confirmed. Again, lengthy feeding experiments on heifers carried out by Hart and his associates [1911] offer little support for the theory. They found, in the case of different groups of animals, fed on rations of different cereals but all having free access to salt, that the amount of sodium chloride consumed bore no relationship to the K content of the ration, either when used alone or when supplemented by K salts.

(2) *Effect on general metabolism.*

(*a*) *Calcium assimilation.* In the emphasis which Bunge lays on the Na : K ratio of the diet, and on the effect which changes in this ratio have on the excretion, he takes into account only the dependence of Na and K on each other, and their effect on Cl. But the possibility must not be overlooked that whether or not Bunge's theory is justified, an excessive proportion of K to Na in a ration may have deleterious effects in other directions. This aspect of the matter also seems to have received little attention, judging by the scanty references in the literature. That an excessive K : Na ratio may have a bad effect on Ca assimilation, and thus be the cause of rickets, was suggested by Seemann [1879] and the idea was later supported by Zander [1881]. Aron [1905] also considered it probable that such a ratio interfered with the deposit of Ca and bone formation in cattle, while Zuntz [1912] has recorded that fodders which cause rickets usually show an excessive proportion of K to Na. Adler [1906], on the ground of metabolic experiments carried out on children, considered that Aron's results were not confirmed for human beings, but interpretation of his results is difficult on account of the shortness of the periods, and of the wide variations in the rations used in the different periods. In two of the three experiments reported his results seem rather to support than to contradict Aron's theory, and in the remaining experiment he altered the acid-base ratio of the diet as well as the Na : K ratio by giving the salts as potassium carbonate and sodium chloride, thus making a change which may have had an important effect on the Ca retention.

(*b*) *Growth and general condition.* Of the general effect on growth and welfare of high K feeding, a few experiments recently recorded are conflicting in their results. Sasaki [1924], in his feeding experiments on rats, found 1·5 : 1 to be the optimum ratio for K : Na in the foodstuffs. If the excess of K over Na were greater than this, the animals could not grow perfectly. Miller [1923], however, has stated that a diet containing K and Na in the ratio of 14 : 1 had no deleterious effect on the growth of young rats. St John [1923], in his recent experiments to which we have already made reference, found that excess of K caused loss in weight and prevented reproduction. In the series of experiments carried out in conjunction with Olson [1925], in which the percentage of Na varied from 0·228 to 2·58, we are informed by one of the authors[1] that the percentage of K in the ration was 0·32. From these figures we find that the proportion of K to Na in the ration which gave the most successful results was 0·6 : 1, while a ration in which K : Na was 1·4 : 1 did not give normal growth or successful reproduction. On the other hand, increasing the amount of Na still further led to deleterious effects on growth and reproduction. This series of experiments brings out clearly the importance for growth of suitable adjustment of the Na : K ratio.

In a recently published paper, Redina [1926] attempts to apply Loeb's theory of ion-antagonism to feeding experiments on adult mice, with special reference to the ions Na, K and Ca. He finds that the proportions of Na and K in the diet have a distinct effect on the weight-curves of the animals, but as he works with an unbalanced diet, lacking in fat and carbohydrate as well as minerals, the significance of his experiments with regard to ordinary metabolic work is not easy to determine.

So far as can be ascertained no systematic attempt has been made since the time of Bunge to deal with the question of the Na : K ratio in any of these aspects until the subject was taken up by the present authors and concurrently by Miller [1923–1926], whose work will be referred to later.

In our earlier paper [1924] we recorded experiments in which we investigated the effect, on the metabolism of the growing pig, of increasing the proportion of Na to K by the addition of Na salts to a cereal ration. In those experiments it was found that the addition of sodium chloride or sodium citrate led to increased assimilation and retention of N, Ca and P. It is of interest to note that during the experimental periods in which this increased retention took place, the ratios of K : Na were 1·62 : 1 and 1·53 : 1 respectively, figures which approximate closely to the ratio of 1·5 : 1 given by Sasaki as the optimum found in his growth experiments. It was found further that while the increased Na intake did lead to increased urinary excretion of K as found by Bunge, this increase was accompanied by a diminution in faecal K, with the result that the balance showed little deviation from the normal. Thus, Bunge's theory with regard to loss of K caused by increased Na intake did not hold in our experiments on growing pigs.

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[1] We are indebted to Mr J. L. St John for his courtesy in supplying this information.

PRESENT INVESTIGATION.

The experiments now to be reported, which have been carried out at intervals from 1923–1926, extend this investigation by seeking to determine the effect of increasing the proportion of K to Na by the addition of potassium citrate to the ration. The investigation has thus a twofold object: (1) to determine whether Bunge's theory, in the aspect in which it is generally applied, viz. sodium chloride impoverishment by excessive K intake, is applicable in the case of the young growing pig, and (2) to find whether the increase in the K intake affects in any way the assimilation and retention of N, Ca and P.

EXPERIMENTAL.

Methods. The experiments were carried out on young pigs (3–4 months old) in the manner previously described. The excretions and balances were determined for N, P, Cl, Ca, Na and K.

Basal diet. As in the previous investigation, the basal diet used in the two experiments to be described consisted of maize, oatmeal, barley-meal and blood-meal, in the quantities given below, with various salt additions as shown.

Daily food intake.

Kind of food	Pig I (control) (g.)	Pig II (g.)	Pigs III and IV (g.)
Maize	500	400	300
Oatmeal	500	400	300
Barley-meal	500	400	300
Blood-meal	50	40	30
$CaCO_3$	27·5	22	7·2
Ca phosphate	18	14·4	—
$CaCl_2$ solution (about 5 % CaO)	—	—	30 cc.
NaCl solution (approx. 20 %)	25 cc.	20 cc.	—

In each experiment the Ca content of the ration was increased by addition of Ca salts. As in the previous experiments, the animals received 10 cc. olive oil daily to assist regular defaecation and in all cases they were given distilled water *ad lib.* In Exp. 1 the ration was further supplemented by addition of calcium phosphate and sodium chloride, in order to make the composition of the ration, with respect to minerals, approximate more closely to that of sow's milk. In Exp. 2 the ration was made exactly similar in composition to that used in the previously reported sodium citrate experiment.

Average composition of the foodstuffs.

	Total N %	P_2O_5 %	Cl %	CaO %	Na_2O %	K_2O %
Maize	1·620	0·658	0·050	0·020	0·184	0·431
Oatmeal	2·130	1·074	0·061	0·087	0·125	0·528
Barley-meal	1·801	0·834	0·110	0·062	0·103	0·648
Blood-meal	13·423	0·315	1·098	0·173	0·987	0·331

The exact intakes of N, P, Cl, etc., in each experiment are stated in the tables giving the experimental data.

Arrangement of experiments. In Exp. 1, pig I was kept as a control, the diet remaining constant for 6 weeks. Pig II, after a preliminary period of 12 days, had potassium citrate added to the ration to the amount of 64 g. daily. On the addition of this large dose of potassium citrate the pig refused to eat its complete ration for 2 days, and the K salt was therefore added in smaller amounts, gradually working up to the full dose. Thus for pig II there is a transition period of 6 days at the beginning of the experimental period (days 13–18) for which the results, being somewhat uncertain, are not included in the table. After 14 days on the full amount of potassium citrate, there followed a post-period of 18 days during which the animal received only the original ration.

In Exp. 2 both pigs were used as experimental animals. After a preliminary period of 14 days for pig III and 10 days for pig IV, each pig received 15 g. potassium citrate for 1 day, and thereafter the full dose of 30 g. daily for 15 days, after which the basal ration was fed for a post-period of 12 days.

The results of the analyses are given in Tables I–IV, and in part in Figs. 1 and 2.

DISCUSSION OF RESULTS.

I. *Effect on general metabolism.* From these experiments, and from a comparison with the results previously recorded for the Na feeding experiments, it would appear that the administration of potassium citrate has had an adverse effect on the assimilation of N, P and Ca (see Fig. 1), although the response of the individual animals to the introduction of the salt varied somewhat in degree, *e.g.* pig III seemed to be slower than pig IV in showing the effect of the potassium citrate, and to recover more quickly when the salt was withdrawn. In all cases the introduction of potassium citrate had a marked diuretic effect.

Table I. *Exp.* 1. *Pig I* (*control*).

Average daily excretions and balances in g.
Duration of experiment: 42 days.
K : Na in food intake = 2·0 : 1 throughout.

Days	Nitrogen					P_2O_5				
(inclusive)	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–7	35·05	12·55	10·09	22·64	12·41	20·23	0·25	11·87	12·12	8·11
8–14	35·05	13·17	9·19	22·36	12·69	20·23	0·27	12·28	12·55	7·68
15–21	34·80	13·21	9·21	22·42	12·38	20·23	0·22	12·35	12·57	7·66
22–28	34·48	13·81	8·18	21·99	12·49	20·25	0·21	12·34	12·55	7·70
29–35	34·48	13·75	8·38	22·13	12·35	20·25	0·27	12·78	13·05	7·20
36–42	34·48	13·77	8·22	21·99	12·49	20·25	0·26	12·68	12·94	7·31
			Cl					CaO		
1–7	4·74	4·08	0·37	4·45	0·29	22·94	1·04	13·16	14·20	8·74
8–14	4·74	3·73	0·23	3·96	0·78	22·94	1·06	13·40	14·46	8·48
15–21	4·74	3·89	0·24	4·13	0·61	22·96	0·87	13·56	14·43	8·53
22–28	4·74	3·60	0·25	3·85	0·89	22·99	0·68	14·07	14·75	8·24
29–35	4·74	3·82	0·25	4·07	0·67	22·99	0·74	14·06	14·80	8·19
36–42	4·74	3·70	0·27	3·97	0·77	22·99	0·58	14·45	15·03	7·96
			Na_2O					K_2O		
1–7	4·78	2·07	1·82	3·89	0·89	8·38	4·34	1·97	6·31	2·07
8–14	4·78	1·94	1·49	3·43	1·35	8·38	4·39	1·67	6·06	2·32
15–21	4·81	1·95	1·27	3·22	1·59	8·36	4·44	1·81	6·25	2·11
22–28	4·86	1·72	1·36	3·08	1·78	8·35	4·55	1·56	6·11	2·24
29–35	4·86	1·58	1·38	2·96	1·90	8·35	4·75	1·55	6·30	2·05
36–42	4·86	1·47	1·31	2·78	2·08	8·35	4·64	1·64	6 28	2·07

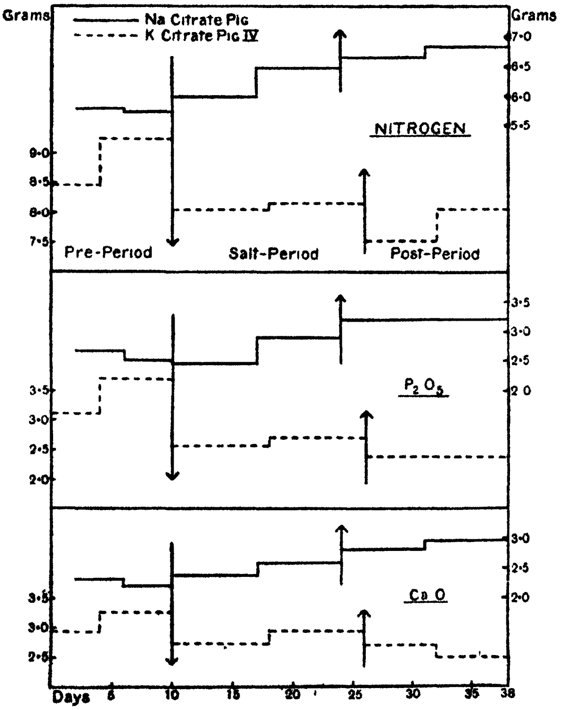

Fig. 1. Nitrogen, P_2O_5, and CaO balances for pigs receiving Na citrate and K citrate respectively, showing the opposite effects of these salts on the general metabolism.

Table II. *Exp.* 1. *Pig II.*

Average daily excretions and balances in g.
Duration of experiment: 50 days.
{K : Na in basal ration = 2·0 : 1.
{K : Na in experimental period = 10·5 : 1.

Days (inclusive)	Nitrogen					P_2O_5				
	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–6	28·04	9·78	7·97	17·75	10·29	16·18	0·20	10·96	11·16	5·02
7–12	28·04	10·36	7·93	18·29	9·75	16·18	0·23	10·72	10·95	5·23
⟶										
*19–25	28·04	11·58	7·07	18·65	9·39	16·18	0·20	11·32	11·52	4·66
26–32	28·04	11·44	7·37	18·81	9·23	16·18	0·26	11·78	12·04	4·14
⟵										
33–39	27·71	11·23	7·13	18·36	9·35	16·19	0·20	11·99	12·19	4·00
40–46	27·58	11·05	7·10	18·15	9·43	16·20	0·20	12·53	12·73	3·47
47–50	27·58	11·22	6·73	17·95	9·63	16·20	0·19	12·92	13·11	3·09
			Cl					CaO		
1–6	3·79	3·21	0·29	3·50	0·29	18·35	0·92	12·22	13·14	5·21
7–12	3·79	2·82	0·24	3·06	0·73	18·35	1·65	10·97	12·62	5·73
⟶										
19–25	3·79	2·99	0·26	3·25	0·54	18·35	0·70	13·12	13·82	4·53
26–32	3·79	3·09	0·26	3·35	0·44	18·35	0·78	13·14	13·92	4·43
33–39	3·79	2·90	0·24	3·14	0·65	18·38	1·27	12·85	14·12	4·26
⟵										
40–46	3·79	2·93	0·28	3·21	0·58	18·39	1·09	13·49	14·58	3·81
47–50	3·79	2·79	0·23	3·02	0·77	18·39	0·77	14·30	15·07	3·32
			Na_2O					K_2O		
1–6	3·82	1·88	1·80	3·68	0·14	6·70	2·81	2·19	5·00	1·70
7–12	3·82	1·95	1·67	3·62	0·20	6·70	2·99	1·89	4·88	1·82
⟶										
19–25	3·82	0·90	1·67	2·57	1·25	35·75	29·53	3·34	32·87	2·88
26–32	3·82	0·73	1·17	1·90	1·92	35·75	29·65	3·54	33·19	2·56
⟵										
33–39	3·87	1·96	1·20	3·16	0·71	6·68	3·27	2·10	5·37	1·31
40–46	3·88	2·08	1·52	3·60	0·28	6·68	2·92	2·16	5·08	1·60
47–50	3·88	1·62	1·53	3·15	0·73	6·68	2·97	2·08	5·05	1·63

⟶ Indicates the introduction of potassium citrate.
⟵ Indicates the withdrawal of potassium citrate.
* Days 13–18 omitted. See text.

Nitrogen. In all three pigs the potassium citrate feeding resulted in increased urinary excretion of N, and this increase accounted almost entirely for the diminished N balance. Only in the case of pig IV was the adverse effect on the balance partially due to increase in the faecal N. It is well known [cf. Orr, 1914] that the diuresis consequent on excessive water ingestion leads to increased urinary excretion of N, hence in the present experiments it is probable that the increase in urinary N may be ascribed to the diuresis brought about by the K feeding. This is borne out by the fact that for pig III, which seemed to be less susceptible in general than pig IV to the K salt, it was found that the diuretic effect was also less marked. It is possible, therefore, that the use of natural feeding stuffs containing a high proportion of K, if they caused no diuresis, might have little effect on the N retention.

Phosphorus. With regard to P excretion, in pigs II and IV, in spite of the diuresis, the K feeding had practically no effect on the urinary P, the diminution in balance being due to increased faecal excretion. On the other hand, pig III showed a slight decrease at first in faecal P, and the diminished balance in the second week of the K feeding was due to an increase in urinary P.

Table III. *Exp.* 2. *Pig III.*

Average daily excretions and balances in g.
Duration of experiment: 42 days.
K : Na in basal ration = 3·0 : 1.
K : Na in experimental period = 11·6 : 1.

Days			Nitrogen					P_2O_5		
(inclusive)	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–6	20·41	7·73	4·72	12·45	7·96	8·31	0·15	5·87	6·02	2·29
7–14	20·41	7·22	4·60	11·82	8·59	8·31	0·22	5·78	6·00	2·31
⟶										
15–22	20·41	7·57	4·44	12·01	8·40	8·31	0·31	5·51	5·82	2·49
23–30	20·41	8·38	4·31	12·69	7·72	8·31	0·58	5·75	6·33	1·98
⟵										
31–36	20·41	8·03	4·12	12·15	8·26	8·31	0·40	5·73	6·13	2·18
37–42	20·41	8·31	4·05	12·36	8·05	8·31	0·55	6·02	6·57	1·74
			Cl					CaO		
1–6	2·97	2·62	0·09	2·71	0·26	6·00	0·29	3·53	3·82	2·18
7–14	2·97	2·52	0·12	2·64	0·33	6·00	0·24	3·41	3·65	2·35
⟶										
15–22	2·97	2·52	0·14	2·66	0·31	6·00	0·18	3·18	3·36	2·64
23–30	2·97	2·55	0·16	2·71	0·26	6·00	0·20	3·66	3·86	2·14
⟵										
31–36	2·97	2·56	0·13	2·69	0·28	6·00	0·19	3·67	3·86	2·14
37–42	2·97	2·46	0·13	2·59	0·38	6·00	0·23	4·08	4·31	1·69
			Na_2O					K_2O		
1–6	1·79	0·57	0·52	1·09	0·70	4·81	1·19	2·71	3·90	0·91
7–14	1·79	0·63	0·43	1·06	0·73	4·81	1·29	2·50	3·79	1·02
⟶										
15–22	1·79	0·22	0·78	1·00	0·79	17·63	12·21	3·32	15·53	2·10
23–30	1·79	0·14	0·61	0·75	1·04	18·49	13·88	2·96	16·84	1·65
⟵										
31–36	1·79	0·48	0·53	1·01	0·78	4·81	1·89	2·23	4·12	0·69
37–42	1·79	0·40	0·22	0·62	1·17	4·81	1·40	2·56	3·96	0·85

⟶ Indicates introduction of potassium citrate.
⟵ Indicates withdrawal of potassium citrate.

Calcium. The Ca results agree for all three pigs. In every case, following an increased K intake, there was an increase in the faecal excretion of Ca, accompanied by a diminution in urinary Ca, resulting in a diminished retention. This is exactly the converse of the results obtained in the sodium citrate experiment. In that case the increased Ca balance was accompanied by increased urinary excretion, the amount of urinary Ca increasing progressively during the whole 14 day period of the sodium citrate feeding, a result which seemed to point clearly to increased assimilation of Ca. The figures now reported therefore, taken in conjunction with those of the sodium citrate experiment, seem to indicate that the Na : K ratio of a diet may have a very considerable influence on the Ca assimilation, and they form an interesting justification of the suggestions put forward by Seemann, Zander, Aron and Zuntz, to which reference has already been made.

Chlorine. The present experiments show, if we consider the two-day results, that there is a very distinct effect of the increased K intake on the urinary Cl excretion. The increase of K intake causes increased urinary excretion of Cl and a negative Cl balance for the first two-day period after the intake. This increase is, however, only transitory, being followed by a decreased excretion for the next 2 days, and, if we consider the average figures for the

Table IV. *Exp.* 2. *Pig IV.*

Average daily excretions and balances in g.
Duration of experiment: 38 days.
{K : Na in basal ration = 3·0 : 1.
{K : Na in experimental period = 11·6 : 1.

Days (inclusive)			Nitrogen					P$_2$O$_5$		
	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
1–4	20·41	7·23	4·71	11·94	8·47	8·31	0·25	4·96	5·21	3·10
5–10	20·41	7·00	4·15	11·15	9·26	8·31	0·18	4·45	4·63	3·68
——→										
11–18	20·41	7·42	4·95	12·37	8·04	8·31	0·17	5·59	5·76	2·55
19–26	20·41	7·51	4·76	12·27	8·14	8·31	0·30	5·32	5·62	2·69
←——										
27–32	20·41	8·59	4·31	12·90	7·51	8·31	0·19	5·75	5·94	2·37
33–38	20·41	8·02	4·32	12·34	8·07	8·31	0·28	5·66	5·94	2·37
			Cl					CaO		
1–4	2·97	2·19	0·21	2·40	0·57	6·00	0·28	2·79	3·07	2·93
5–10	2·97	2·41	0·22	2·63	0·34	6·00	0·29	2·46	2·75	3·25
——→										
11–18	2·97	2·51	0·13	2·64	0·33	6·00	0·14	3·13	3·27	2·73
19–26	2·97	2·55	0·14	2·69	0·28	6·00	0·15	2·92	3·07	2·93
←——										
27–32	2·97	2·62	0·09	2·71	0·26	6·00	0·17	3·14	3·31	2·69
33–38	2·97	2·51	0·14	2·65	0·32	6·00	0·19	3·32	3·51	2·49
			Na$_2$O					K$_2$O		
1–4	1·79	0·62	0·48	1·10	0·69	4·81	1·31	2·16	3·47	1·34
5–10	1·79	0·69	0·40	1·09	0·70	4·81	1·09	2·19	3·28	1·53
——→										
11–18	1·79	0·34	0·99	1·33	0·46	17·63	12·27	3·08	15·35	2·28
19–26	1·79	0·30	0·96	1·26	0·53	18·49	13·23	2·99	16·22	2·27
←——										
27–32	1·79	0·67	0·65	1·32	0·47	4·81	1·78	2·26	4·04	0·77
33–38	1·79	0·58	0·19	0·77	1·02	4·81	1·48	2·30	3·78	1·03

——→ Indicates the introduction of potassium citrate.
←—— Indicates the withdrawal of potassium citrate.

periods, it is found that continued excessive intake of K salts has little effect on excretions and balances. When the K salt is withdrawn there is a reverse transitory effect. Urinary Cl diminishes temporarily, but the organism adjusts itself at once. This transitory influence of increased K intake on urinary Cl is perhaps the most decided effect observed in all the potassium citrate experiments, although again the response of the animals to the change in diet varies in degree. Thus pig III showed a less decided effect when the K salt was withdrawn than pigs II and IV. In pig II the effects are exaggerated by the large dose of K salt. Table V and Fig. 2 show this effect of increased K intake on the urinary excretion of Cl, the figures being two-day excretions.

A comparison of these figures with those of the sodium citrate experiment shows that sodium citrate has the reverse effect on urinary Cl excretion, a point which was not brought out in the previous paper. Table VI and Fig. 2 give these results for the excretions and balances of Cl[1], using the corrected intake.

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[1] A correction has to be made in the Cl balance figures as previously published for the Na experiments. More recent work by two of us [Husband and Godden, 1927] on the method of determining Cl in foodstuffs has shown that the method then employed gave results considerably too low in the case of cereal foods. Making this correction for the intake of Cl it will be found that the pigs instead of showing a steady negative balance were really on a small positive balance.

Table V.

	Pig II		Pig III		Pig IV	
	Urinary Cl	Cl balance	Urinary Cl	Cl balance	Urinary Cl	Cl balance
Average for last 8 days of pre-period	5·79	+1·30	5·03	+0·66	4·59	+0·90
Days 1 and 2 of potassium citrate period	9·70	Large neg. balance[1]	5·96	−0·30	5·83	−0·29
Days 3 and 4 of potassium citrate period	0·50	+6·85	4·10	+1·59	4·21	+1·49
Last 8 days of potassium citrate period	6·15	+0·92	5·10	+0·51	5·10	+0·55
Days 1 and 2 of post-period	4·15	+2·97	4·95	+0·73	4·70	−1·05
Days 3 and 4 of post-period	7·47	−0·38	5·22	+0·49	5·83	−0·10

[1] The exact figure here is uncertain, as the animal did not eat the complete ration for 2 days on account of the large dose of potassium citrate, but it may be taken as approximately −4·3.

Table VI.

	Urinary Cl	Cl balance
Last 8 days of pre-period	5·05	+0·46
Days 1 and 2 of sodium citrate period	4·29	+1·30
Days 3 and 4 of sodium citrate period	5·64	−0·17
Last 8 days of sodium citrate period	5·01	+0·51
Days 1 and 2 of post-period	5·26	+0·30
Days 3 and 4 of post-period	5·26	+0·34
Days 5 and 6 of post-period	4·96	+0·65

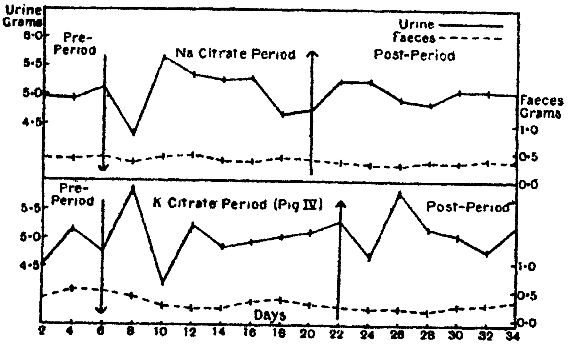

Fig. 2. Urinary and faecal excretions of chlorine for pigs receiving Na citrate and K citrate respectively, showing the opposite effects of these salts on the urinary excretion of chlorine, and the temporary nature of the disturbance.

II. *Bunge's theory of chlorine impoverishment by potassium intake.* It will be seen from a study of the above potassium citrate experiments that while the Cl results agree with those of Bunge in his experiments on himself with regard to the *temporary* increase in Cl excretion caused by increased K intake, they offer no justification of his theory that continued high intake of K may cause impoverishment of the organism in Cl. The immediate increase in urinary Cl is followed at once by decreased excretion when the K salt is withdrawn (see Tables V and VII), as Bunge himself found. We find further that this fall below normal occurs even when the high K intake is continued. From the experimental data available it seems clear that the theory of Cl impoverishment caused by high K intake is quite untenable. On the contrary, the results obtained bring out very strikingly the rapidity with which the organism can adjust itself to sudden changes in the diet, and demonstrate clearly its ability to maintain its Cl balance in spite of large variations in the Na : K ratio of the diet.

Potassium. The increased K intake resulted in an increased K balance for all three pigs, but the excess retained during this period was got rid of in the post-period through the urine, by which also the major portion of the ingested potassium citrate was voided during the experimental period.

Sodium. While the results obtained for the Na excretions and balances in these experiments are not so consistent or so decisive in their nature as those for Cl, yet they cannot be regarded as offering any support for Bunge's theory of Na impoverishment caused by excessive K intake. In considering the results it seems advisable to point out that, owing to the method of Na estimation, the interpretation of small variations in the results is less certain than is the case with other estimations. Since the Na is estimated by difference, it follows that when large amounts of K salt are present in the urine, and consequently small amounts of material have to be taken for analysis, as is the case in the potassium citrate periods, the possibility of error in the final Na result is greatly increased. Hence only *considerable* variations in the Na figures can be accepted as forming any basis for argument.

(*a*) *Urinary sodium excretion.* Although with regard to Cl the figures we have obtained confirm Bunge's findings of a *transient* increase in urinary excretion consequent on increased K intake, we have not obtained the same confirmation as regards the excretion of Na. On only two occasions have we observed a suggestion of such increase. (1) In the transition period of Exp. 1, where the results were however somewhat vitiated by the fact that the large dose of potassium citrate caused pig II to go off its food, the urinary Na increased somewhat for the first 2 days of the period, the daily average being 1·9 g. for the last 2 days of the pre-period, and 2·2 g. for the first 2 days of the experimental period. This small difference is, however, in no way inconsistent with the two-daily variations found during the pre-period, particularly when it is remembered that the animal was not eating well. (2) Another experiment, not otherwise recorded in this paper, was conducted to throw further light on this particular point, attention being paid solely to the intake and excretion of Na and K. The urinary Na for the first day of the K period was 0·51 g. Na_2O as against an average of 0·39 g. for the 8 days of the pre-period. As the excretion for the last 2 days of the pre-period had been rather below normal, this slight increase, while it *may* be due to increased K intake, is again quite in keeping with the ordinary daily fluctuations. In this case the average urinary excretion of Na_2O for 8 days of continued high K feeding was only 0·43 g., as compared with the pre-period average of 0·39 g., and the Na balance for the period showed a slight improvement in spite of the slight increase in urinary excretion.

In no other case did we find even this amount of support of Bunge's findings. Urinary excretion of Na diminished rather than increased during the K-feeding periods, and even when the K salt was given again for 1 day only to pigs III and IV (Table VII), at the end of the post-period recorded in Tables III and IV, no increase in urinary Na was observable, though the Cl effect already referred to was clearly shown.

Table VII.

	Pig III				Pig IV			
	Na_2O		Cl		Na_2O		Cl	
	Urine	Bal.	Urine	Bal.	Urine	Bal.	Urine	Bal.
Daily average for last 6 days of post-period	0·40	1·17	2·46	+0·38	0·58	1·02	2·51	+0·32
Potassium citrate day	0·06	1·30	3·01	−0·18	0·21	1·07	3·12	−0·24
Day after potassium citrate	0·26	1·18	1·33	+1·56	0·35	1·19	1·48	+1·41

(*b*) *Sodium balance.* As regards the Na balance also, the weight of evidence is against Bunge's theory of Na impoverishment by K feeding. In Exp. 2 the decrease in urinary Na for the K period was accompanied by increased faecal excretion of Na (cf. the results of the Na experiments already published in which increased urinary K and decreased faecal K go together). With pig III, as also with pig II, and with the animal in the short experiment already briefly mentioned, there was an improvement in the Na balance for the experimental period, while with pig IV the diminution in balance was due to the largely increased faecal excretion, which more than counterbalanced the diminished urinary excretion. Thus our results do not bear out Bunge's theory of loss of Na from the organism through increased urinary excretion. It must be borne in mind, however, that these experiments were conducted on young growing animals, while Bunge's experiments with an adult human being as subject were carried out under widely different experimental conditions.

Miller [1923–1926] has recently carried out a series of interesting experiments on "Potassium in animal nutrition," to which we have already made some reference. In his first paper, dealing with the influence of K on urinary Na and Cl excretion in pigs, Miller claimed that his results, using a starch diet, a synthetic ration, or a milk diet, agreed with those of Bunge in showing that a sudden increase of K salts in the diet caused an immediate increase in Na and Cl excretion for the following 24-hour period. He found further that the Na and Cl excretion decreased when the high K intake was continued.

From a close study of Miller's data we are unable at times to reconcile the figures in the tables with the conclusions he draws from them, particularly with regard to the effect on Na excretion. Thus in Table II, Nov. 4th, on the starch diet, there is no increase in Na excretion on giving the K salt, although the Cl figure shows the expected increase. In Table VII, on the synthetic ration, there is little or no Na increase for any of the dates on which the K salt was introduced, while in Table IX, giving the results for a pig on milk diet, there is not only no initial increase in Na excretion, but the figures show a distinct decrease for the whole K period. It may be mentioned here that the Cl figures in Table IX agree very closely with those we have obtained both with regard to the initial rise and fall at the beginning of the K period, and also as regards the sudden drop and subsequent increase when the K salt is withdrawn.

Other instances can be found in the tables which do not fit in with Miller's general conclusions, and it seems as if Miller were ready to accept Bunge's statements in preference to the evidence of his own experimental results, instead of recognising how often the two are in conflict. We are inclined therefore to accept the results of Miller's experiments on pigs, which it must be remembered covered only urinary excretions and were not balance experiments, as offering additional support to our conclusions, instead of regarding them as a confirmation of Bunge's results. His later metabolic experiments, in which rats were used as subjects, are scarcely comparable either with his or our experiments on young pigs, or with those of Bunge on himself.

SUMMARY.

1. The addition of potassium citrate to a ration of cereal grain fed to growing pigs, led to decreased assimilation and retention of nitrogen, phosphorus and calcium.

2. The increase of potassium salts in the ration caused an immediate increase in the urinary excretion of chlorine, followed at once by a decrease, after which continued high intake of potassium had little or no effect on the excretion. The withdrawal of the potassium salt, conversely, caused an immediate drop in urinary chlorine, followed by an increase.

3. The results obtained for growing pigs with regard to sodium excretion do not support the theory of sodium impoverishment put forward by Bunge as the result of experiments on himself.

4. For the growing animal it would appear that any adverse effect of excessive potassium content in a ration is due not to impoverishment of the organism in sodium or chlorine, but more probably to the depressing influence on the metabolism of nitrogen, phosphorus, and calcium.

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CXXXI. THE TOXICITY OF PHENYLARSINIC ACIDS FOR *BALANTIDIUM COLI* MALM. IN CULTURES, IN RELATION TO THEIR CHEMICAL CONSTITUTION.

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(*Received June 25th, 1927.*)

In the course of an examination into the effect of a variety of drugs on cultures of *Balantidium coli* it was found that out of three organic arsenic compounds used, only two showed any considerable degree of toxicity. As this seemed to indicate that a relationship might exist between toxicity and chemical constitution, the effect of a series of selected arsenicals was examined *in vitro*. The results obtained appear to be of sufficient interest to merit a brief record.

Since the discovery of the nature of atoxyl by Ehrlich in 1907, numerous papers have appeared on the use of organic arsenic compounds for protozoal diseases. Many derivatives of both ter- and quinque-valent arsenic compounds have been employed in these researches, but as yet little has been brought to light regarding the laws correlating toxicity and chemical constitution. In this connection a noteworthy feature is that different results may be obtained in toxicity tests on organisms according to whether the experiments are carried out *in vivo* or *in vitro*. Thus while sodium *p*-aminophenylarsinate has proved to be a most effective remedy for sleeping sickness, *in vitro* it is almost without action on trypanosomes.

In order to explain this discrepancy between *in vitro* and *in vivo* experiments, it has been suggested that the arsenicals undergo some change in the animal body, and that it is the resulting product and not the original substance which displays toxicity. These divergencies, however, may be more apparent than real, for toxicity tests on microscope slides may have little significance—this has been clearly shown in the case of *E. histolytica* by Dobell and Laidlaw [1926, 1]—and although trypanosomes can be readily cultivated they are in cultures so far removed from their natural conditions that little weight would attach to results from drugs tested in this way. In the present investigation this difficulty does not appear to be so marked, for it has been found possible to grow *B. coli* under conditions which seem to approximate to natural ones very closely. Particulars of this will shortly be published elsewhere.

Technique.

The strains of *B. coli* used for this work were obtained from the caecum of pigs. So far as can be determined from morphological evidence this is the same ciliate as is found in man, in which host it causes an intractable dysentery. It is possible, however, that there are really two species in the pig as described by McDonald [1922]—*B. coli* and *B. suis*—but in the material used for the toxicity tests the *B. coli* form predominated and in any case both forms are affected by drugs in exactly the same way so that we shall regard the cultures as being those of *B. coli*.

Material from the caecum of freshly killed pigs was planted in Dobell and Laidlaw's [1926, 2] medium consisting of an inspissated horse-serum slope covered with Ringer solution containing the whites of two eggs to one litre of solution. In addition a small quantity of sterile rice starch is essential. In this medium *B. coli* grows with great freedom and shows no signs of degeneration—a feature so common in the cultivation of protozoa. Indeed it seems probable that *in vitro* results on intestinal protozoa grown in this medium are much more reliable than is the case with blood-protozoa on account of the more nearly natural conditions obtained in the authors' tubes. No strain was used for experimental purposes until growth had been well maintained for several weeks.

1 % solutions of the drugs were made in distilled water and the higher dilutions, for use in the toxicity experiments, were prepared by dilution with the requisite quantity of Ringer egg-white solution. In cases where dilutions lower than 0·1 % were required, a 1 % solution was made up in the Ringer egg-white solution. In this way, the liquid medium was never diluted to any appreciable extent by addition of the drug solution. About 5–6 cc. of fluid medium containing the required drug were used in each tube and to this one drop of inoculum, obtained from a mixture of healthy 24 and 48 hour cultures was added. The tubes were incubated at 37° and examined on the second and third days after inoculation, growth in cultures always being at its best on these days. The effect of the drug was estimated by observing the numbers of living and dead organisms, the increase or decrease in these numbers, and especially by the condition of the organisms. such as the rate of ciliary movement, the size and shape of the body and the presence or absence of degenerative changes in the endoplasm. A record of life alone in cultures is not sufficient, as, in two cultures showing living organisms, one might be quite normal and the other degenerate. For this reason the tables showing our findings are annotated to show, as far as possible, the condition of the organisms in the culture tubes. Control tubes are not given in the tables in order to economise space but in all experiments at least one control tube was put up for every four drug solution tubes and usually one control for three drug solution tubes. No experiment is recorded in which the controls were unsatisfactory.

Table I. *Numbers of living* (L) *and dead* (D) *cultures on 3rd day of incubation.*

	Dilutions	1 : 100		1 : 250		1 : 500		1 : 750		1 : 1000		1 : 3000		1 : 5000		1 : 7500	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
A	Sodium *o*-hydroxyphenylarsinate	—	—	—	—	—	—	—	—	0	3	—	—	—	—	—	—
B	Sodium *o*-aminophenylarsinate	—	—	—	—	—	—	—	—	0	3	—	—	—	—	—	—
C	Sodium *p*-aminophenylarsinate (Atoxyl)	—	—	—	—	—	—	—	—	0	3	—	—	0	4	0	9
D	Sodium *p*-hydroxyphenylarsinate	—	—	—	—	—	—	—	—	0	7	—	—	0	3	—	—
E	Sodium *m*-aminophenylarsinate	—	—	—	—	—	—	—	—	0	8	—	—	0	5	—	—
F	Sodium 3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Cyclosan)	—	—	—	—	0	5	0	5	0	14	4*	2	8	6	3	2
G	Sodium *N*-glycineamidophenylarsinate (Tryparsamide)	—	—	—	—	0	10	1	10	1	13	7*	7	10	3	7	0
H	Sodium 4-hydroxy-5-acetylaminophenylarsinate (Sodium Stovarsol)	0	5	0	4	1	6	1	7	9†	3	5	0	3	0	—	—
J	Sodium 2-hydroxy-5-acetylaminophenylarsinate (Sodium Troposan)	3‡	0	3‡	0	5	0	5	0	5	0	—	—	—	—	—	—
K	Sodium 8-acetylamino-3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Sodium Parosan)	3‡	0	3	0	5	0	5	0	6	0	—	—	—	—	—	—

	Dilutions	1:10,000		1:15,000		1:20,000		1:30,000		1:40,000		1:50,000		1:60,000		1:70,000	
		L	D	L	D	L	D	L	D	L	D	L	D	L	D	L	D
A	Sodium *o*-hydroxyphenylarsinate	0	4	—	—	0	4	1§	3	3§	9	6	3	5	0	3	0
B	Sodium *o*-aminophenylarsinate	0	4	—	—	0	4	1§	3	0	4	5	3	5	0	4	0
C	Sodium *p*-aminophenylarsinate (Atoxyl)	4*	9	3	8	5	11	6	3	7	0	6	0	3	0	—	—
D	Sodium *p*-hydroxyphenylarsinate	0	8	0	4	5	2	7	0	4	0	4	0	—	—	—	—
E	Sodium *m*-aminophenylarsinate	0	9	3†	8	4	3	6	0	4	0	—	—	—	—	—	—
F	Sodium 3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Cyclosan)	6	0	6	0	—	—	—	—	—	—	—	—	—	—	—	—
G	Sodium *N*-glycineamidophenylarsinate (Tryparsamide)	3	0	6	0	—	—	—	—	—	—	—	—	—	—	—	
H	Sodium 4-hydroxy-5-acetylaminophenylarsinate (Sodium Stovarsol)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
J	Sodium 2-hydroxy-5-acetylaminophenylarsinate (Sodium Troposan)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
K	Sodium 8-acetylamino-3-hydroxy-1 : 4-benzisoxazine-6-arsinate (Sodium Parosan)	—	—	—	—	—	—	—	—	—	—	—			—	—	—

* Organisms few and sluggish.
† Organisms sluggish
‡ Slightly deformed.
§ Organisms few and degenerate.

THE RELATION BETWEEN TOXICITY AND CHEMICAL CONSTITUTION.

Of the recent work on the relation between toxicity and chemical constitution of organic arsenic compounds, researches on trypanosomes have claimed by far the greater part. In this connection reference may be made to papers by Fourneau and his collaborators [1923, 1926], King and Murch [1925], Hewitt and King [1926], and Ewins and Everett [1927]. Although it seems probable that the relation between toxicity and structure may depend to a certain extent on the specific organism experimented upon, one or two generalisations appear to be permissible from the work on trypanosomes.

(1) The aminophenylarsinic acids show considerable toxicity but the position of the amino-group is of importance, the *ortho*-isomer being most highly toxic.

(2) Marked toxicity is exhibited by the hydroxyphenylarsinic acids but the actual position of the hydroxyl grouping is less important.

(3) Fourneau found that in the treatment of trypanosomiasis, the trypanocidal power of *p*-aminophenylarsinic acid was not diminished by acetylation, but under the same treatment, the *meta*- and *ortho*-isomers showed decreased toxicity.

The compounds considered in the present investigation were examined as sodium salts and fall into three groups.

(*a*) Amino- and hydroxy-phenylarsinates: toxic to *B. coli* in dilutions above 1/20,000.

(*b*) Cyclosan, tryparsamide and stovarsol: toxic in dilutions below 1/10,000.

(*c*) Troposan and parosan: not toxic in dilutions of 1/500 and only very slightly so in 1 % dilutions.

It will be seen from our tables that the results obtained by *in vitro* work on a species of intestinal protozoa are to some extent in agreement with the generalisations given above for *in vivo* work on trypanosomes.

(1) The aminophenylarsinates show considerable toxicity, the *ortho*-isomer being most highly toxic. The position of the amino-group has a very definite effect.

(2) The substitution of a hydroxy-group in place of the amino-group does not greatly alter the toxicity, unless the degree of hydration is taken into account—see below—when the *ortho*-hydroxy salt is the most toxic examined. The position of the group seems to have a considerable effect on toxicity.

(3) Of cyclosan and tryparsamide little can be said beyond that, in the latter case, substitution in the amino-group of *p*-aminophenylarsinate has lowered the toxicity considerably.

(4) In all cases examined, addition of an acetylamino-group in a position *meta* to the arsenic grouping has resulted in a marked decrease in the parasiticidal effect. This is most marked in troposan where, by addition of the $CH_3.CO.NH$-grouping, *o*-hydroxyphenylarsinate has been rendered non-toxic.

All the toxic sodium phenylarsinates dealt with in this paper, with the exception of sodium *o*-aminophenylarsinate, are hydrated, and, when the degree of hydration is taken into account, the figures given for the lowest dilution at which the salt is not toxic are changed to those given in Table II.

Table II.

	A	*B*	*C*	*D*	*E*	*F*	*G*	*H*
Lowest dilution at which the salt is completely non-toxic	$\frac{1}{73,500}$	$\frac{1}{60,000}$	$\frac{1}{55,000}$	$\frac{1}{41,250}$	$\frac{1}{38,800}$	$\frac{1}{11,800}$	$\frac{1}{7,727}$	$\frac{1}{3,920}$
Molecules of water	3	0	5	5	3	3	½	5

After making this correction certain points are clarified. In Table I *A* and *B* appear to be nearly equally toxic with a slight balance in favour of greater toxicity on the side of the amino-salt. The same is true of *D* and *E*. In Table II, on the other hand, there are well-defined intervals between the different toxic points and the *ortho*-hydroxy salt is definitely the more toxic.

The order of the toxicity of the compounds, however, still remains as given above. Previous workers do not appear to have taken the matter of hydration into account.

We wish to express our indebtedness to Dr A. J. Ewins, of Messrs May and Baker, Ltd., for a gift of most of the chemicals used in the investigation and for much useful information.

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CXXXII. THE EFFECT OF ALKALOSIS ON THE EXCRETION OF CHLORIDE AND ON CARBOHYDRATE METABOLISM.

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THE phenomena accompanying alkalosis have received considerable attention in recent years, both because of their clinical bearing in such cases as tetany and because of certain peculiar consequences of theoretical interest.

Apart from its occurrence in pathological conditions alkalosis may be induced by two main methods in the normal subject: (*a*) by overbreathing, (*b*) by administration of alkalis. The former method is a severe one for the subject, as if it is persisted in for any length of time marked symptoms of tetany are produced. The expulsion of CO_2 from the alveoli leads to an uncompensated alkalosis and the p_H of the blood may change markedly as the attempt to compensate becomes futile. The attempt to compensate consists in lowering the volume of expired air and the excretion of alkali in the urine. In addition, there occurs an excretion of ketonic substances in the urine.

In inducing alkalosis by the administration of alkalis the effects produced are much milder because the compensation of the condition is much more effective. Large doses of sodium bicarbonate or acetate may be taken without any obvious disturbance other than an occasional diarrhoea and, of course, marked thirst. The compensatory mechanism again consists in diminished breathing, retention of CO_2 in the alveoli and excretion of large quantities of alkali in the urine.

No symptoms of tetany have been seen in the experiments in this paper.

Of course, in a case of lowered kidney efficiency, a flooding of the tissues with alkali without rapid excretion may bring about tetanic symptoms. Such cases are on record.

As in the case of alkalosis following overbreathing, the alkalosis following alkali administration is sometimes followed by ketosis.

The production of ketosis in alkalosis has been explained in two principal ways: (1) that the alkali in being excreted carries with it the ketonic acids from the seat of their formation, that is a so-called "washing-out" effect; (2) that it is due to a disturbance of carbohydrate metabolism. It is clear that the determination of the correct view is of the first importance in justifying or condemning alkali therapy in diabetes, or other conditions accompanied by acidosis.

In this paper the method of inducing alkalosis was by administration of alkali by mouth. The questions which are considered are the following.

(1) What is the rate of excretion of sodium bicarbonate in normal man after different doses?

(2) Under what conditions is the alkalosis accompanied by ketosis?

(3) The effect of alkalosis on ketosis in starvation.

(4) The effect of alkalosis on carbohydrate metabolism.

(5) The effect of alkalosis on the action of insulin.

(6) The effect on glycogen formation.

The methods of analysis were the following.

(1) For the determination of bicarbonate in the urine the apparatus of Van Slyke for blood-gas analysis was used. The urine was diluted so that 1 cc. gave not more than about 0·7 cc. of CO_2 on treatment with acid. The volume of CO_2 was found by direct absorption in normal NaOH, corrected and expressed as $NaHCO_3$.

(2) Urine- and blood-chlorides were determined by Van Slyke's method.

(3) Blood-sugar estimations were made by MacLean's method. Urine-sugar was estimated by Shaffer-Hartmann's or Bertrand's method.

(4) Glycogen estimations were made by Pflüger's method. The animals used were white rats. They were killed by a smart blow on the head and immediate decapitation. Violent reflex spasms occurred for a few seconds. The liver was quickly removed, dropped into a weighed wide-mouthed flask and quickly weighed to the nearest centigram. With a little practice this can be done so quickly that the loss of water vapour is negligible.

The final filtrate was always made up to 100 cc. and 25 cc. were hydrolysed for 3 hours with HCl, the glucose being estimated by Bertrand's method.

While the liver was being weighed the carcase was rapidly skinned, eviscerated, weighed, minced, and about 40 g. worked up for glycogen. The final filtrate was made up to 100 cc. and 25 or 50 cc. were hydrolysed.

(5) Phosphates in the urine were determined by Briggs' modification of the Bell-Doisy method.

(6) Respiratory quotients were determined by means of the Douglas bag and the Haldane apparatus.

(7) Alveolar CO_2 values were obtained by averaging the analyses of the inspiratory and expiratory samples.

Exp. 1. The excretion of $NaHCO_3$ in a normal man. The subject was the writer. A meal had been taken at 9.30 a.m. At 12 noon 10 g. $NaHCO_3$ in 100 cc. water were ingested.

No disturbance of any kind was experienced and there was no ketosis. The concentration of $NaHCO_3$ in the urine reached a maximum in 2 hours and remained practically constant for the following 2 hours. The total excretion of alkali in 4½ hours was 2·58 g. and 8 hours later the urine was still strongly alkaline to litmus. The excretion of NaCl must be compared with the ordinary excretion after the morning meal.

	Urine				
		NaHCO$_3$ g.		NaCl g.	
Time	Vol. cc.	%	Total per hour	%	Total per hour
11.47 a.m.	57	—	—	1·485	—
12.00 noon	6	—	—	1·695	0·471
12.30 p.m.	24	0·135	0·065	1·594	0·766
1.00 ,,	43·5	0·842	0·732	1·083	0·942
1.30 ,,	42	0·914	0·768	0·960	0·806
2.00 ,,	42	1·021	0·858	0·906	0·762
2.30 ,,	32	1·093	0·700	0·854	0·546
3.00 ,,	26	1·111	0·578	0·765	0·398
3.30 ,,	26·5	1·111	0·589	0·765	0·404
4·30 ,,	44	1·052	0·463	0·800	0·352

Exp. 2. The excretion of chloride and inorganic phosphate after the morning meal. Breakfast 9.30 a.m. 100 cc. water at 12.20 p.m. Subject M. W. G.

	Urine				
		NaCl g.		Inorganic phosphate mg. P	
Time 11.20 a.m.–	Vol. cc.	%	Total per hour	%	Total per hour
12.20 p.m.	52	1·465	0·762	26·64	13·85
1.20 ,,	58	1·646	0·955	17·30	10·03
2.20 ,,	50	1·571	0·789	48·62	24·31
3.20 ,,	56	1·594	0·893	57·81	32·37
4.20 ,,	47	1·566	0·736	56·42	26·52
5.20 ,,	39	1·472	0·574	64·66	25·22

It is now seen that although the kidney was slowly excreting NaHCO$_3$ in Exp. 1 it was beginning to find difficulty in excreting chloride. This will be seen much more markedly in the next experiment.

Exp. 3. The excretion of NaHCO$_3$, NaCl and inorganic P after three doses of NaHCO$_3$ of 10 g. each at 11.38 a.m., 12.33 p.m., 1.35 p.m.; each dose in 40 cc. water. Breakfast at 9.30 a.m.

	Urine						
		NaHCO$_3$ g.		NaCl g.		Inorg. P mg.	
Time	Vol. cc.	%	Total per hour	%	Total per hour	%	Total per hour
11–11.30 a.m.	31	—	—	1·46	0·90	32·3	20·64
12.30 p.m.	68	0·466	0·317	1·30	0·88	12·8	8·70
1.30 ,,	53	1·606	0·851	0·56	0·30	30·9	32·75
2.30 ,,	91	1·820	1·656	0·268	0·24	34·6	31·49
3.30 ,,	117	2·998	3·508	0·218	0·26	23·4	27·38
4.30 ,,	90	2·922	2·630	0·206	0·19	26·4	23·76
5·30 ,,	54	2·656	1·434	0·150	0·08	38·7	20·90
6.30 ,,	44	2·427	1·068	—	—	56·7	24·95
7·20 ,,	22	1·898	0·500	—	—	80·0	17·6

In this experiment about 12 g. of NaHCO$_3$ were excreted in 7 hours and the urine was still alkaline at noon the following day.

There was no ketosis throughout this experiment and the compensation of the alkalosis was perfect. The marked fall in the output of chloride was very striking and has been observed in every experiment in which the kidney was made to excrete large amounts of alkali. This fall may be variously interpreted and without entering into a discussion on the matter on this occasion we may briefly say that there seem to be two possible views.

(1) The kidney may only be capable of excreting up to a certain limiting concentration of ions, which might be calculable from a summation of the molar concentrations of each electrolyte in the urine multiplied by its ionisation coefficient. The work of Adolph [1923] in this connection is suggestive.

(2) Although we have given our chloride figures as NaCl it must be remembered that after all what is estimated is chlorine ions, *i.e.* negative ions. It may be that the retention of chlorine is a compensatory mechanism to deal with the alkalosis.

No appreciable fall in the excretion of inorganic phosphate has been found in alkalosis. Evidence has also been obtained that there is no interference with the excretion of inorganic sulphate.

The following experiment was carried out to see if any evidence of retention of chlorine in the blood could be obtained in alkalosis.

Exp. 4. Breakfast 9.15 a.m. 30 g. $NaHCO_3$ in 150 cc. water at 1.15 p.m. Blood taken from arm vein for blood- and plasma-chlorides, collected under oil.

	Urine						Blood	
			$NaHCO_3$ g.		NaCl g.		NaCl g. %	
Time 10.15 a.m –	Vol. cc.	Ketones	%	Total per hour	%	Total per hour	Whole blood	Plasma
1.15 p.m.	166	–	—	—	1·715	0·949	0·478	0·598
2.15 ,,	75	–	0·888	0·666	1·030	0·773	0·447	0·579
3.40 ,,	170	∓	1·844	2·213	0·399	0·479	0·471	0·603
4.55 ,,	164	+	2·814	3·692	0·297	0·389	—	—
5.30 ,,	63	+	2·407	2·600	0·220	0·238	—	—
8.15 ,,	217	+	3·240	2·557	0·208	0·164	—	—
9.55 ,,	125	∓	1·815	1·361	0·246	0·185	—	—

In the 8⅔ hours of this experiment the total excretion of $NaHCO_3$ was 19·23 g., the maximum hourly excretion being 3·69 g. It will be observed that the mild ketosis appeared when the concentration of alkali in the urine was 1·84 %. This ketosis was associated with only 4–5 mg. % of acetone. This became more marked as the excretion of alkali continued but at 9.55 p.m. when the concentration of bicarbonate was 1·815 % the reaction was only just perceptible. This matter will be returned to later. The chloride excretion as before fell very markedly, but the blood chlorides, whilst indicating some initial dilution of the blood, soon returned to the original level both in whole blood and plasma.

After a dose of 30 g. of bicarbonate one very soon feels a large accumulation of fluid in the intestine. This fluid is fairly certainly rich in chloride which probably makes a large call on the store of salt in the body. The blood-chloride is, as we have seen, kept up to normal levels and it appears that the kidney is responsible for this by refusing to excrete chloride in these conditions at the same rate as it would normally.

In the next experiment a period of starvation was undergone before inducing alkalosis in order to see how far the diminution in chloride excretion could be pushed.

Exp. 5. (*a*) Chloride excretion in a normal man after 13½ hours' starvation. 100 cc. water at 11.30 a.m. Subject M. W. G.

	Urine		
		NaCl g.	
Time	Vol. cc.	%	Total per hour
9–11.30 a.m.	86	1·280	0·440
12.30 p.m.	41	1·371	0·562
1.30 ,,	53	1·443	0·765
2.30 ,,	42	1·226	0·515
3.30 ,,	34	1·191	0·405
4.30 ,,	23	0·938	0·216
5.30 ,,	23	0·764	0·176

(*b*) Effect of bicarbonate ingestion on chloride excretion in a normal man after 14 hours' starvation. Subject M. W. G. 25 g. $NaHCO_3$ in 100 cc. water at 12 noon.

	Urine					
			NaCl g.		$NaHCO_3$ g.	
Time	Vol. cc.	Rothera test	%	Total per hour	%	Total per hour
12.00 noon	78	–	1·006	0·392	—	—
1.00 p.m.	51	–	0·934	0·476	0·575	0·293
2.00 ,,	88	–	0·430	0·378	1·978	1·741
3.00 ,,	68	–	0·340	0·231	2·427	1·650
4.00 ,,	50	–	0·278	0·139	2·445	1·223
5.00 ,,	46	–	0·245	0·113	2·481	1·141
5.45 ,,	32	+	0·232	0·099	2·660	1·135

The fall in chloride excretion in (*b*) is very marked and levels are reached which ordinarily are only attained after 40 hours' starvation. In this experiment the total excretion of alkali was 7·2 g. in 6 hours and the urine was alkaline late the following day. A definite ketosis appeared 5¾ hours after the $NaHCO_3$ was taken, when there was 2·66 % in the urine. The intensity of the ketosis was such as to make it very unlikely that it was due to a "washing-out" effect. In Exp. 4 the ketosis was more intense when the urine contained 1·84 % $NaHCO_3$ than when in the above experiment the urinary alkali was 2·44 %.

In fact it seems that the more rapidly the alkali is excreted the less likelihood is there that a ketosis will appear.

Further, the value of the very small quantities of ketones excreted in these conditions is negligible as a neutralising mechanism.

In Exp. 5 (*b*) the minimum hourly output of chloride is seen to be 0·099 g. The following experiment was carried out to see if values below this could be obtained by increasing the period of starvation.

Exp. 6. (*a*) The excretion of chloride after 38 hours' starvation. Subject M. W. G. 100 cc. water at 8 a.m.

	Urine		
		NaCl g.	
Time	Vol. cc.	%	Total per hour
9.05–10.00 a.m.	24	0·412	0·108
11.10 a.m.	29	0·483	0·120
11.45 "	16	0·395	0·108
12.00 noon	7	0·412	0·116
12.30 p.m.	12	0·412	0·099
1.00 "	13	0·332	0·086
1.30 "	13	0·365	0·095
2.00 "	16	0·355	0·114
2.30 "	13	0·254	0·066
2.45 "	9	0·294	0·106
3.15 "	15	0·370	0·111

The hourly excretion of chloride is seen to be very steady during the 6 hours of the experiment, but this is not always the case. In the following table the chloride output is much higher but the dose of $NaHCO_3$ brings it down to minimal levels in about 2 hours.

(*b*) A fast of 41 hours was undergone and 20 g. of $NaHCO_3$ in 100 cc. water were taken at 11.30 a.m. Subject M. W. G.

	Urine				
		NaCl g.		$NaHCO_3$ g.	
Time	Vol. cc.	%	Total per hour	%	Total per hour
9.30 a.m.	188	0·295	0·052	—	—
11.00 "	52·5	0·781	0·273	—	—
11.30 "	14·5	0·797	0·231	—	—
12.00 noon	17	0·720	0·245	—	—
12.35 p.m.	32	0·510	0·280	0·950	0·54
1.08 "	51	0·369	0·342	1·127	1·045
1.15–1.45 p.m.	30	0·217	0·130	1·118	0·671
2.10 "	31	0·126	0·094	1·478	1·100
2.41 "	35·5	—	—	2·084	1·480
3.11 "	42	0·152	0·128	2·264	1·902
3.35 "	24	0·171	0·103	2·290	1·374

It is seen that even after 41 hours of complete fasting and when the kidney in addition is excreting as much as 1·9 g. $NaHCO_3$ per hour the output of chloride is still at the rate of about 0·1 g. per hour. This minimum is rarely diminished and it would appear that the concentration at which the alkali is excreted is limited by this minimum of chloride. Summation of the molar concentrations of the bicarbonate and chloride within certain limits gave figures of some constancy in particular experiments.

For example, the following figures were obtained in one case after 30 g. $NaHCO_3$: 0·027, 0·029, 0·033, 0·034, 0·026, 0·023, 0·024, 0·016.

These values were not corrected for ionisation. The failure in constancy occurred generally during the fall in bicarbonate excretion, the recovery in the chloride being much slower than the fall in the alkali.

Exp. 7. Seeing that the ingestion of alkali brings about a markedly diminished excretion of chloride, it was of interest to see if a similar effect could be demonstrated in the stomach.

Mr W. J. Griffiths of this laboratory, who is very expert in gastric analysis, kindly undertook to be the subject of this experiment.

The experiment was started 4½ hours after a moderate breakfast. The stomach-tube was passed and the stomach washed out until the washings were clear. 25 g. of $NaHCO_3$ were taken in 100 cc. water at 1 p.m. Gastric and urinary samples were taken about every 15 minutes.

	Gastric contents			Urine		
Time	Reaction to Congo red	% NaCl	Time 8.30 a.m.–	Vol. cc.	NaCl g. per hour	$NaHCO_3$ g. per hour
1.15 p.m.	Neg.	0·317	1.00 p.m.	316	0·621	0·020
1.30 ,,	Neg.	0·317	1·15 ,,	55	1·335	0·033
1.45 ,,	Neg.	0·311	1·30 ,,	21	0·454	0·063
2.00 ,,	Neg.	0·355	1·47 ,,	82·5	1·243	0·833
2.15 ,,	Neg.	0·437	2.00 ,,	13·5	0·298	0·703
2.34 ,,	Neg.	0·466	2.15 ,,	37	0·789	1·751
2.45 ,,	Neg.	0·484	2.34 ,,	40	0·440	1·760
3.00 ,,	Neg.	—	2.45 ,,	33	0·363	1·760
3.15 ,,	Neg.	0·640	3.00 ,,	26·5	0·389	1·698
3.30 ,,	Neg.	0·638	3.15 ,,	22	0·303	1·293
3.45 ,,	Acid	—	3.30 ,,	—	—	—
4.15 ,,	Acid	—	—	—	—	—

The steady rise in chloride in the stomach, accompanied by a fall in the urinary chlorides, makes it clear that whatever the mechanism for sparing chloride may be the gastric glands do not assist in its maintenance. We may further regard these values of stomach chlorides as corroborating the conclusion that the supply of chlorine from the blood is not interfered with.

THE EFFECT OF ALKALOSIS ON CARBOHYDRATE METABOLISM AND ON KETOSIS.

It was shown by Haldane and his co-workers [1924] that alkalosis had some inhibitory effect on carbohydrate metabolism. Alkalosis, whether induced by overbreathing or alkali ingestion, lowers the normal tolerance to carbohydrate, that is to say, that doses normally producing a good rise in R.Q. and not followed by glycosuria gave rise in alkalosis to glycosuria and practically no change in the respiratory metabolism. Accompanying these phenomena, markedly abnormal blood-sugar curves were found.

The following experiments were designed to throw further light on this question.

Exp. 1. This experiment was carried out to see if the mere presence of alkali in the gut affected sugar metabolism. 5½ hours after a light meal 25 g. of $NaHCO_3$ in 100 cc. water were ingested and 14 minutes later 50 g. of glucose were taken. Subject M. W. G. Alkali at 2.46 p.m. Sugar at 3 p.m.

Time	Blood-sugar %	Urine		NaHCO$_3$ g.	
		Vol. cc.	Rothera test	%	Total per hour
2.20 p.m.	—	181	-	—	—
2.45 „	0·087	—	-	—	—
3.00 „	0·090	12	-	0·009	0·001
3.30 „	0·141	19	-	0·119	0·025
4.00 „	0·135	33	-	0·779	0·514
4.30 „	0·090	45	-	1·383	1·224

The blood-sugar values of this subject in analogous conditions without alkali are of the following order: 0·102, 0·150, 0·125, 0·095, 0·090 %. Thus it appears that the mere administration of alkali and an almost immediate ingestion of sugar do not give rise to an abnormal blood-sugar curve; nor was there any appearance of sugar in the urine. The situation is, however, quite different if a period of some hours is allowed to elapse between the taking of the alkali and the sugar. This was done in the next experiment.

Exp. 2. The conditions were similar to those in Exp. 1. 30 g. NaHCO$_3$ were taken at 12 noon and 50 g. glucose at 3.35 p.m. Subject M. W. G.

Time	Blood-sugar %	Urine				
		Time	Vol. cc.	Rothera test	Glucose g. %	NaHCO$_3$ g. %
12.00 noon	0·133	—	—	—	—	—
1.30 p.m.	0·115	1.32 p.m.	285	-	—	1·341
3.10 „	0·102	1.50	—	-	—	—
4.5 „	0·146	2.35	113	±	—	2·019
4.35 „	0·171	3.30	76	+	0·034	2·430
5.05 „	0·120	4.07	38	+	0·034	2·502
5.35 „	0·090	4.37	23	+	0·466	1·841
		5.10	34	+	0·140	1·573
		5.41	—	±	0·070	1·090

The blood-sugar curve here produced is clearly of the so-called lag type. There is a smart rise above the threshold and a rapid readjustment to normal levels. With larger doses of alkali the curve can be made to reach higher levels and the return to resting values can be delayed by some hours. Traces of reducing substances were present in the urine between 5.10 and 5.41 p.m. when the blood-sugar was certainly below 0·12 %. Some slight effect on the threshold value of glucose seems to have been produced but it is difficult to be sure about this.

It is to be observed that the storage of sugar was in full activity at a time when 1·84 % of NaHCO$_3$ was being excreted by the kidney. Further, throughout the whole experiment only 6·4 g. of alkali were excreted, so that, even allowing for the loss which certainly takes place by the bowel, there was still a great deal being absorbed.

It would appear then that the interference with sugar storage takes place most markedly at a time when the excretion of alkali is at a maximum. The effect can pass off rapidly if the removal of the alkali *via* the kidney is sufficiently quick to prevent any accumulation of it above a certain optimum level in the tissues. It is also seen that a definite ketosis appeared in spite of the administered glucose. This ketosis reached a maximum when the interference with adequate sugar metabolism was most pronounced and almost disappeared as the excess alkali was thrown out.

There can be little doubt that alkalosis when well established produces a definite diminution in the power to store sugar. In the next experiment evidence is presented that there is also some interference with oxidation as indicated by respiratory quotient determinations.

Exp. 3. The effect of alkalosis in a case of markedly increased sugar tolerance. Subject M.

The case chosen was a eunuchoid whose sugar tolerance had been examined on two previous occasions. On the first occasion, 19. iii. 1926, 50 g. of glucose 12 hours after food gave rise to the following blood-sugar values at half-hourly intervals: 0·075, 0·104, 0·113, 0·091, 0·085 %. On the second occasion, 22. ii. 1927, the R.Q. values were also obtained:

Blood-sugar %	0·072	0·100	0·104	0·113	0·081
R.Q.	0·88	1·01	1·02	0·99	0·95

On 1. iii. 1927, 40 g. $NaHCO_3$ were given at 11.0 a.m. and 50 g. glucose at 12.30 p.m. The conditions were similar to those in the previous tests.

Time ...	10.30	11.30	12.00	12.30	1.00	1.15	1.30	2.00	2.30	3.00	3.30
Blood-sugar %	0·085	0·090	0·095	0·106	0·135	0·122	0·120	0·120	0·133	0·111	—
R.Q. ...	0·86	—	0·91	—	0·92	—	0·876	0·895	—	—	—

Such blood-sugar values have never been found in this patient under ordinary conditions and there can be little doubt that the storage mechanism for sugar has in some way been rendered less active than normally. Further, the rise in R.Q. was much below that which occurred in the control experiment and we must suppose that oxidation was interfered with, although by no means completely inhibited.

Exp. 4. The effect of alkalosis in a case of severe glycosuria without ketosis. Subject Mrs V.

The case chosen was a woman of 50 years weighing 15 st. 10 lb. and still gaining weight slowly. A dose of 75 g. of glucose 14 hours after food gave the following blood-sugar values at half-hourly intervals: 0·336, 0·327, 0·400, 0·376, 0·312 %, and in the 2½ hours of the experiment the urine contained 24·5 g. glucose.

This patient constantly excreted between 100 and 120 g. sugar a day even on a restricted diet. She had no ketosis at any time during 12 months' intermittent examination. It must apparently be supposed that in this type of case adequate fat metabolism is maintained by means of carbohydrate formed from either fat or protein or both, and that this is more readily utilisable than ordinary exogenous carbohydrate.

In this experiment a light breakfast was taken by the patient at 8 a.m. and a dose of 30 g. of $NaHCO_3$ was given at 11.38 a.m.

	Urine						
Time	Vol. cc.	Sugar g. %	Total sugar (g.)	Rothera test	NaCl g. per hour	$NaHCO_3$ g. %	R.Q.
10.55 a.m.	185	5·83	10·79	–	0·335	—	0·74
11.35 „	152	8·25	12·54	–	0·748	—	—
11.38 „	30 g. $NaHCO_3$ in 100 cc. water						
1.00 p.m.	400	4·70	18·80	+	1·028	0·523	0·70
2.07 „	282	3·30	9·31	+	0·488	1·125	0·72
3.05 „	173	3·15	5·45	+	0·230	1·079	—
4.05 „	142	—	—	+	0·145	1·033	0·70
5.00 „	78	—	—	+	0·083	0·888	—

The blood-sugar values were:

Time	11.20	12.00	2.05	3.00	4.00	4.50
Blood-sugar %	0·457	0·434	0·347	0·349	0·304	0·316

In 4½ hours 56·89 g. of sugar were excreted after only a very light meal at 8 a.m., but it is seen that even with 8·25 % of glucose in the urine there was no trace of ketonic acids. Judging from the R.Q. very little sugar was being metabolised, but even this little was thrown out of action by the alkali and a ketosis was established 1½ hours after the $NaHCO_3$ was taken. Still it must be observed that the amount of ketones excreted was not such as one would associate with a complete inability to metabolise sugar. Anti-ketogenic substances must still have been present and in action.

The total bicarbonate excreted was 9·28 g. in 5½ hours which is not less rapid than in the normal subject. It is further to be observed that the excretion of sugar was not interfered with.

As in the previous experiments there occurred a striking fall in the urinary chlorides.

THE EFFECT OF ALKALOSIS ON THE KETOSIS OF STARVATION.

Administration of alkali to diabetics leads to an increased excretion of ketonic acids. The same thing occurs in the ketosis of starvation in normal subjects. The ketosis induced in rats by feeding on a high fat diet is much increased by alkali feeding. Lactic acid may also be increased in the urine in these conditions and it was tentatively suggested by Macleod and Knapp [1919] that these acids might be of importance in the regulation of blood reaction. As a matter of fact the quantities in which these acids are produced are so small that they can only be of very little value as a neutralising mechanism. In our view the increase in ketosis in alkalosis is an expression of an interference with carbohydrate metabolism.

The following experiment is regarded as strong evidence in favour of this view.

Exp. 5. The writer underwent a period of 41 hours' starvation after which a marked ketosis was present. It had been found on many previous occasions that such a ketosis can be completely destroyed by 50 g. glucose in about 1 hour and always in less than 1¼ hours [Goldblatt, 1925].

In this experiment 20 g. $NaHCO_3$ were taken at 11.30 a.m. and 50 g. glucose at 12.45 p.m.

Time	Blood-sugar g. %	Urine Vol. cc.	Urine $NaHCO_3$ g. %	Urine Glucose g. %	Urine Acetone mg. per hour	R.Q.	Alveolar CO_2 %	Acetone in expired air per 2 mins. mg.
9.30 a.m.	—	188	—	—	10·60	—	—	—
10.12 ,,	0·075	—	—	—	—	0·67	—	0·286
10.30 ,,	—	—	—	—	—	—	5·585	—
10.45 ,,	0·076	—	—	—	—	—	—	—
11.00 ,,	—	52·5	—	—	35·70	—	—	0·182
11.20 ,,	—	—	—	—	—	—	5·655	—
11.30 ,,	—	14·5	—	—	24·36	—	—	—
11.46 ,,	—	—	—	—	—	0·68	—	—
12.00 ,,	0·077	17	—	—	24·62	—	—	—
12.20 ,,	—	—	—	—	—	—	6·075	0·092
12.35 ,,	—	32	0·95	—	45·42	—	—	—
1.05 p.m.	0·113	—	—	—	—	0·64	—	0·068
1.08 ,,	—	51	1·127	—	—	—	—	
1.17 ,,	0·164	—	—	—	43·40	—	—	0·077
1.22 ,,	0·175	—	—	—		—	—	—
1.45 ,,	0·187	30	1·118	0·061	23·40	0·74	5·983	—
2.02 ,,	0·209	—	—	—	—	—	—	0·120
2.10 ,,	—	31	1·478	0·400	—	—	—	—
2.17 ,,	0·168	—	—	—	—	—	—	—
2.35 ,,	0·115	—	—	—	—	—	—	—
3.11 ,,	0·078	—	2·264	—	23·00	—	—	—

The findings in this experiment may be summarised as follows.

(1) The already diminished tolerance to sugar existing after starvation is accentuated in the alkalosed starving subject.

(2) The ketosis was most markedly increased during the second hour after the alkali was taken and when the concentration of $NaHCO_3$ in the urine was only about 1 %.

(3) The ketosis had not disappeared and was only slightly diminished 9 hours after the glucose was taken. In the table only the figures up to 2½ hours after the sugar are given, but the nitroprusside test was still strongly positive 6½ hours after this.

(4) The excretion of ketonic substances in the breath is greatly diminished under the influence of alkali.

(5) The rise in alveolar CO_2 is brought in as a compensatory effect and reaches a maximum long before the kidney reaches its maximum excretory function. The alveolar mechanism appears to be the first line of defence until the kidney gets rid of the excess bicarbonate. This fact is shown very well in the following table of an experiment in which the writer took 30 g. $NaHCO_3$ about 3 hours after a meal.

Time	11.48	12.45	1.13	1.55	2.35	3.20	4.57	5.40
Alveolar CO_2 % ...	5·37	5·58	5·89	6·38	—	6·45	5·94	—
Urinary $NaHCO_3$ g. %	0	0·744	1·822	2·564	2·676	2·137	3·243	2·764

The evidence presented in the foregoing experiments leaves little doubt that alkalosis is accompanied by a definite diminution in carbohydrate storage and oxidation. The next logical step seemed to be to find if this effect might be referred back to an interference with the effectiveness of insulin and further, whether the glycogenic function of the liver and tissues was in any way affected.

THE EFFECT OF ALKALOSIS ON THE ACTION OF INSULIN.

For this purpose rabbits and rats have been used. It will be unnecessary to give more than typical experiments. Similar results to my own have been obtained by Hetenyi [1926], who found that the effect of insulin is strongly delayed by alkali given *per os*. He also found that acid given *per os* had no appreciable effect on the action of insulin. The findings with alkali have been confirmed and we give the following experiment on a single rabbit, which was very striking.

This animal, which had been observed on several occasions, could have its blood-sugar lowered to values of 0·02 % without symptoms. As we had never seen such an animal before experiments were repeated several times with the following results.

Exp. 6. Effect of two units of insulin injected subcutaneously on the blood-sugar of the above rabbit. Weight 2 kg. There were no symptoms. No food, of course, was given during these experiments.

Time	2.25	2.40	3.10	4.15	4.45
Blood-sugar g. %	0·096	Insulin 2 units	0·062	0·020	0·020

Exp. 7. Same rabbit as above. Blood taken from veins of both ears.

Time ...	12.30	12.35	1.15	2.10	3.08	3.40	4.35	4.40
Blood-sugar g. % ...	0·135	Insulin 3 units	0·090	0·030	0·020	0·025	0·125	0·122

Exp. 8. In conditions similar to those of Exps. 6 and 7 the same rabbit was given 3·5 g. of $NaHCO_3$ by stomach-tube at 12 o'clock and insulin injected as below.

Time ...	11.20	12.00	12.08	12.55	1.35	2.20
Blood-sugar g. % ...	0·111	0·152	Insulin 4 units	0·177	0·131	0·122
Time ...	2.45	3.20	3.50	4.20	6.00	
Blood-sugar g. % ...	Insulin 4 units	0·072	0·056	0·067	No symptoms	

In this last experiment the initial rise of blood-sugar to 0·152 % was due to the passage of the stomach-tube. The first dose of insulin brought the blood-sugar down to 0·122 % in 2 hours 12 minutes. A further dose of four units only lowered it to 0·067 % in a further 1½ hours, a level which could be attained by two units in ½ hour starting at levels between 0·08 and 0·09 %. When it is considered that altogether eight clinical units were injected, it seems clear that the alkali has in some way definitely inhibited the action of the insulin.

It seems feasible to suppose that a similar sort of thing is occurring in the alkalosed human subject and that here lies the explanation of the intolerance to carbohydrate which accompanies alkalosis.

THE EFFECT OF ALKALOSIS ON GLYCOGEN STORAGE.

For this purpose white rats were used. The recent work of Macleod and co-workers [Barbour, Chaikoff, Macleod and Orr, 1927] on the glycogen distribution in rats under the influence of insulin is of great importance in showing how to obtain uniform controls in these animals. For our experiments, however, it soon became evident that absolutely uniform controls were unnecessary as the changes induced by alkali were remarkable.

White rats of between 130 and 140 g. weight were taken and put on a diet of bread and milk. This diet suits them admirably and good deposits of glycogen are soon laid down both in liver and muscles. After the required period of feeding the animals were weighed, tapped smartly on the head and immediately decapitated. The liver was quickly weighed and worked up for glycogen. The carcase was rapidly skinned, eviscerated, weighed and an aliquot part worked up for glycogen.

Exp. 9. Three rats on bread and milk. No glycosuria. No ketosis.

No.	Days on bread and milk	Weight (g.)	Weight of liver (g.)	Weight of skinned carcase (g.)	Glycogen (mg.)	
					Liver	Carcase
1	8	142	6·28	56	104	36
2	13	135	6·39	60	96	69
3	16	152	6·78	61·5	254	114

Exp. 10. Another four rats which had been on the same diet as those in Exp. 9 were now given $NaHCO_3$ added to their bread and milk. The periods of alkalisation varied from 3 to 8 days. The animals took the bicarbonate well and passed considerable amounts of alkali in the urine. In one case 0·49 g. of $NaHCO_3$ appeared and in another 0·66 g. in one day. The following table gives the results of analysis of these animals.

No.	Days on bread and milk	Days on alkaline diet	Weight (g.)	Weight of liver (g.)	Weight of skinned carcase (g.)	Glycogen (mg.)	
						Liver	Carcase
4	29	3	149	5·83	71	Traces	13
5	33	7	135	4·81	60	Traces	Traces
6	34	8	132	4·86	63	26	Traces

The fourth alkalosed rat was now taken off the alkali and put on an ordinary bread and milk diet for 2 days. On the first day after the return to ordinary diet it excreted alkali but no acetone and there were 48 mg. (as glucose) of Cu-reducing substances in the urine. The urine also reduced alkaline safranine so that sugar was probably present. On the second day the urine was amphoteric, acetone was present and the reduction value was 53 mg. The rat was now killed and analysed.

Exp. 11. Rat no. 7.

Days on alkalised diet	Days of return to ordinary diet	Weight (g.)	Weight of liver (g.)	Weight of skinned carcase (g.)	Glycogen (mg.)	
					Liver	Carcase
8	2	140	7·92	52	156	104

The above Exps. 9, 10 and 11 seem definitely to show that in conditions of powerful alkalosis the animals do not lay down glycogen but the readjustment to normal storage is rapid when the excess alkali is removed.

SUMMARY.

1. Utilising doses of $NaHCO_3$ up to 30 g. the maximum concentration of bicarbonate in the urine of a normal subject is about 3·2 %. The rise in bicarbonate excretion is constantly associated with a very marked fall in chloride excretion. The fall in chloride excretion is not accompanied by a fall in blood- or plasma-chloride. It is difficult to be sure whether this effect is due to an inability of the kidney to excrete chloride or to a diversion of tissue-chloride so that the "head" of chloride is removed.

2. The fall in urinary chlorides in alkalosis is not accompanied by a similar phenomenon in the stomach, where the secretion of chloride may be quite normal.

3. Alkalosis is accompanied by a definite diminution in carbohydrate tolerance and sometimes by a ketosis. The alkalosis-ketosis is not a mere "washing-out" effect, but is rather to be regarded as a result of defective carbohydrate utilisation.

4. The ketosis of starvation is much increased in alkalosis and the maximum excretion of ketonic acids does not necessarily coincide with the maximum output of alkali in the urine.

5. The ketolytic action of glucose in starvation is markedly diminished by alkali.

6. The action of insulin is diminished by alkali given *per os*.

7. The deposition of glycogen in liver and muscle can be greatly diminished by alkalosis.

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CXXXIII. A NOTE ON THE ESTIMATION OF ADRENALINE.

BY LESLIE CHARLES BAKER AND GUY FREDERIC MARRIAN.

From the Department of Physiology and Biochemistry,
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(*Received June 30th, 1927.*)

INTRODUCTION.

DURING the progress of other work in this laboratory it was necessary to carry out quantitative estimations of the adrenaline present in the small adrenal glands of the rat and pigeon. The well-known colorimetric method of Folin, Cannon and Denis [1913], described for estimations on glands of about 2 g. in weight, seemed the most suitable for this purpose.

On adapting this method to the small glands of about 0·03 g. weight that were being used, results were obtained that showed considerably wider variation than was anticipated. At the same time it was noticed that, under the conditions of our adaptation, the rate of colour change of the adrenaline solution differed from that of the uric acid standard solution.

Accordingly it was decided to investigate the effect of such variable factors as temperature on the rate of colour production of solutions of adrenaline, using the glasses of a Lovibond tintometer as a standard against which to match the blue colour.

The possibility of loss of adrenaline due to oxidation in the method of extraction employed by Folin, Cannon and Denis was also examined and found to be small but definite. This loss takes place after the addition of sodium acetate to the hot hydrochloric acid extract and was avoided by using cold trichloroacetic acid as the extracting agent. This acid proved satisfactory in every way and gave a clear protein-free filtrate.

Apparatus.

The best source of light for use with a tintometer is daylight from a north window. However, as we wished to work at any time and during the winter months an artificial source of light was necessary. A stout cardboard box was painted black inside and placed upon its side, which was 42 cm. long. Two daylight lamps were inserted in the end, one above the other and 15 cm. apart, and across the other open end of the box two sheets of the special transparent paper supplied by the Tintometer Company, Salisbury, were placed close together. A third sheet of this paper was inserted about 18 cm. nearer the lamps than the other two sheets. Such an arrangement gave a fairly large and evenly illuminated field and the tintometer was placed at such a distance that the structure of the transparent paper could not be seen. Two small weighing bottles, of internal diameter 2·35 cm., served as cells for holding the solutions to be matched. During the day both daylight lamps were employed but at night it was found easier to match if only one was used. A thermostat was maintained at 20° (or the appropriate working temperature) and held the matching cells and all the reagents and vessels used in producing the blue colour.

Temperature and other factors.

By using a tintometer the blue colour may be matched against a non-variable standard and the difficulty of matching two colours of not quite the same tint, such as those given by adrenaline and uric acid with the phosphotungstic "uric acid reagent," is avoided.

In order to plot time-intensity curves of the blue colour developed, an accurately weighed amount of adrenaline, between 0·03 and 0·04 g., was dissolved in 10 % trichloroacetic acid and the volume made up to 100 cc. with this acid. Small portions of this standard solution, between 1 cc. and 5 cc., were diluted to 25 cc. with 10 % trichloroacetic acid and 3 cc. of such a solution were run into a 25 cc. flask, diluted somewhat with water and placed in the thermostat. In this way the concentration of adrenaline could be readily varied while the acid concentration remained constant. When sufficient time had been allowed for the flask and its contents to reach the appropriate working temperature, 0·5 cc. of the phosphotungstic "uric acid reagent" described by Folin and Trimble [1924] was added, washed in with a little distilled water, the time noted, 5 cc. of a saturated solution of sodium carbonate added, washed in and made up to a final volume of 25 cc. with distilled water. The flask was then shaken briskly and a suitable amount of the solution poured into the matching cell. The blue colour was matched at measured intervals of time after the addition of the sodium carbonate. The amount of adrenaline estimated in these experiments was of the order of that present in 0·03 g. of adrenal gland. It was found that the blue glasses of the tintometer required the admixture of about 10 % of the yellow glasses in order to match the tint produced by adrenaline, but only the value of the blue glasses was recorded. It was difficult to match accurately any solution paler than five or six blue units.

The time-intensity curves for different concentrations of adrenaline were plotted at 20°. These curves, shown in Fig. 1, rise to a maximum in almost exactly 20 minutes and demonstrate clearly that, over the range studied, the concentration of adrenaline does not affect the time of maximum colour development at any one temperature.

The rate of colour development for one concentration of adrenaline at 14°, 16° and 25° was next studied. Fig. 2 shows the curves obtained; clearly, the reaction velocity is greater at higher temperatures, the time taken to reach a maximum increasing as the temperature falls; at 25° the maximum is reached after 10 minutes, at 16° after about 35 minutes and at 14° after 45 minutes. The curves at 25°, 16° and 14° are typical curves selected from a number obtained at those temperatures, while the curve at 20° is an average of all those obtained for that concentration at that temperature.

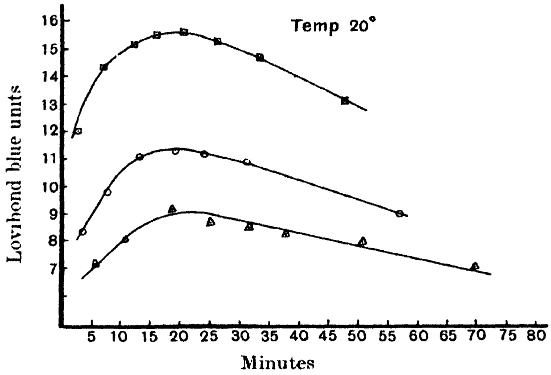

Fig. 1.

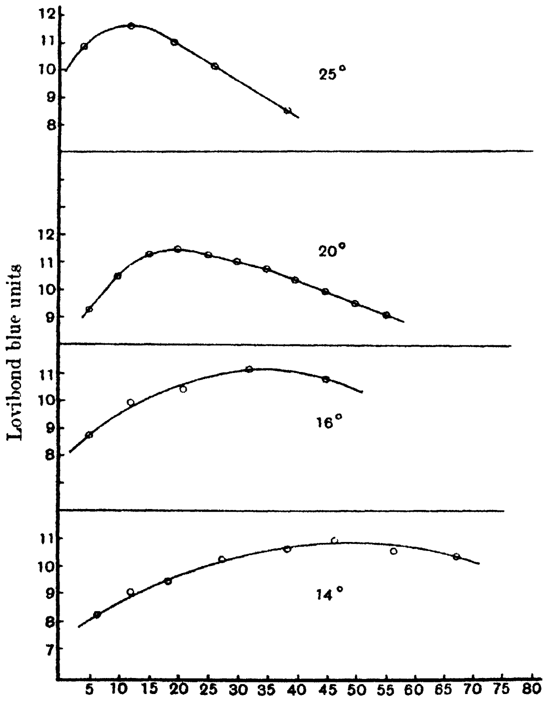

Fig. 2.

In order to estimate adrenaline in solutions of unknown strength, a calibration curve was constructed. Such a curve is of course dependent on the size of cell for matching used and possibly also on the particular specimen of "uric acid reagent" employed and must therefore be independently constructed by each worker. As a working temperature 20°, at which maximum colour develops in 20 minutes, was selected as being the most convenient. The known adrenaline content was plotted against the blue colour developed after 20 minutes. Three such points were taken from the curves in Fig. 1 and two more were obtained from separate determinations, one for a higher and the other for a lower concentration of adrenaline. The calibration curve is a mean straight line drawn through these points. Other known concentrations of adrenaline were then estimated and marked on the graph. From these the possible maximum experimental error was calculated to be 7 %.

Oxidation.

The possibility of loss of adrenaline by oxidation during the boiling in hydrochloric acid solution after addition of sodium acetate, in the method of Folin, Cannon and Denis, was investigated with solutions of pure adrenaline and, except where mentioned below, following their technique exactly. In each experiment about 0·008 g. of adrenaline was used, as this was a fair average of the amount estimated by these workers in 2 g. of adrenal gland. After addition of sodium acetate, in the first experiment the solution was brought to the boil, made up to 100 cc., roughly divided, one half cooled rapidly under the tap and the other half allowed to cool slowly on the bench. 1·25 cc. of one half was then pipetted into a 25 cc. flask and matched in the way described in the last section. 1·25 cc. of the other half was similarly estimated. In the second experiment boiling after addition of sodium acetate was continued for one minute and in the third experiment for two minutes; the subsequent procedure in these two experiments was the same as in the first.

Table I shows that no significant loss takes place if the method of Folin, Cannon and Denis be followed exactly, but that if the boiling be continued for 2 minutes and the solution allowed to cool slowly there is a 10 % loss. Thus when working with quantities of adrenaline a hundred times as small, appreciable loss might readily occur.

Table I.

Exp.	Treatment after addition of NaAc	Units of blue with cooling		Units of blue in control	Loss		% loss	
		Rapid	Slow		Rapid	Slow	Rapid	Slow
1	Brought to boil	12·4	12·0	12·6	0·2	0·6	2·0	5·0
2	Boiled for 1 min.	12·1	11·6	12·6	0·5	1·0	4·0	8·0
3	Boiled for 2 mins.	11·7	11·4	12·6	0·9	1·2	7·5	10·0

Extraction of adrenal glands.

The efficiency of a 10 % aqueous solution of trichloroacetic acid as an extracting agent was investigated. A number of fresh rat adrenals were available.

These were minced finely with scissors to give a fairly homogeneous mash and three samples were weighed out, two of about 0·03 g. and one of 0·06 g. The adrenaline content of one 0·03 g. sample was estimated directly and to each of the other two samples a volume of an adrenaline solution equal to that of a previously estimated volume from the same solution was added. The glands were then ground fine and the adrenaline content of each estimated. The results are set out in Table II. The very slight loss observed shows that trichloroacetic acid is a very suitable extracting agent.

Table II.

Exp.	Weight of gland in g.	Units of blue observed	Control blue units	Blue units adren. pres. calc. from 0·0315 g.	Units of blue calc., i.e. control + calc.	Loss blue units	% loss
1	0·0315	4·9	5	—	—	—	—
2	0·0329	9·9	5	5·1	10·1	0·2	2·0
3	0·0629	14·5	5	9·8	14·8	0·3	2·0

Our extracts were accordingly made in the following way. The adrenal glands, in which the adrenaline was to be estimated, were carefully freed from connective tissue and transferred to a tared pyrex test-tube cut down to half size; this was weighed again. A little purified sand and a few drops of about 10 % trichloroacetic acid solution were added and the gland was finely ground in the tube with a glass rod, 3 cc. of the 10 % trichloroacetic acid were added and the sand and precipitated protein agitated, the sediment was allowed to settle and the liquid filtered through a wad of cotton-wool into a 25 cc. flask. The residue in the tube was washed first with 5 cc. and then with 3 cc. of distilled water plus a few drops of trichloroacetic acid solution, the washings were filtered into the flask and the latter set in the thermostat. The extract was then made up and matched in the manner described in an earlier section. Table III shows some results for a number of normal male rats.

Table III. *Starved 6–8 hours before experiment.*

Rat	Body weight (g.)	Rect. temp. ° C.	Weight adrenal glands (g.)	per 100 g. body weight (g.)	mg. adrenaline	Adrenaline mg per 1 g. gland	Adrenaline mg per 100 g. body weight
1	126	37·7	0·0386	0·0306	0·086	2·23	0·068
2	130	38·1	0·0334	0·0257	0·083	2·49	0·064
3	119	38·2	0·0338	0·0284	0·074	2·19	0·062
4	124	36·6	0·0376	0·0303	0·071	1·89	0·057
5	129	37·3	0·0320	0·0248	0·058	1·83	0·045
Average	126	37·6	0·0337	0·0267	0·074	2·13	0·059

The Authors wish to express their thanks to Prof. J. C. Drummond for much helpful advice and his constant interest in the work. One of them (G. F. M.) is indebted to the Medical Research Council for a personal grant.

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CXXXIV. THE CONSTITUENTS OF *FABIANA IMBRICATA*.

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(*Received June 20th, 1927.*)

THE plant *Fabiana imbricata* (Ruiz and Pavon) or *pichi-pichi* is a well-known shrub growing in South America and belongs to the Solanaceae.

It has been used medicinally for centuries by the Incas, and about 50 years ago attracted some attention in Europe as a specific for urinary and liver complaints.

The first chemical examination appears to have been carried out by Nivière and Lotard [1887], who state definitely that the drug is free from alkaloid. They ascertained the presence of a fluorescent principle, a yellow precipitate with lead acetate analogous to "l'esculo-tannate de plomb," gummy matter, glucose and calcium tartrate.

A more detailed examination was carried out, however, by Kunz-Krause [1899], who found the leaves to contain choline, chrysotropic acid (now known as scopoletin) and a substance which he called "fabioglucotannoid" and for which he suggested the formula:

$$C_6H_{11}O_5.O.C_6H_2(OH)(OMe)CH : CH.CO_2H$$

or its internal anhydride. The compound was described as a yellow, very hygroscopic substance softening at 80° and beginning to swell up at 105°.

Kunz-Krause further isolated from the wood a volatile oil which he called fabianol (no formula given) together with a non-volatile white crystalline solid melting at about 280° and capable of being sublimed. This was called fabianaresen and the formula was $C_{54}H_{90}O_6$ although cryoscopic determinations indicated $C_{18}H_{30}O_2$.

It is evident that Kunz-Krause isolated products of doubtful purity, and the results obtained in the present investigation have confirmed this, more especially in regard to the so-called fabioglucotannoid.

This substance has now been shown to be a new glucoside of scopoletin for which the name *fabiatrin* is proposed, and its properties are described in the experimental part of this paper.

The authors have been unable to confirm the presence of choline, but have indicated the presence of a basic substance which forms a picrate. In addition, a saponin has been detected, and the physiological effect of the plant appears to be due entirely to this substance. Although the drug is classified among the Solanaceae, the absence of alkaloids is confirmed.

EXPERIMENTAL.

The material used in this investigation consisted of twigs about 3 feet in length and varying in diameter from 1 to 2 inches. The cortex was very thin, and the wood extremely hard. The drug possessed a characteristic odour, and a substance (alcoholic) was isolated possessing the odour in a marked degree.

The twigs were very kindly ground for us by Messrs Evans, Lescher and Webb, Ltd., and we would here express our deep appreciation to C. J. S. Sendall, Esq., for the help so readily given.

As a preliminary experiment, 50 g. of the ground material were successively extracted in a Soxhlet apparatus, when the following amounts of extract, dried at 100°, were obtained.

Light petroleum (B.P. 29–45°)	extracted	0·44	%
Ether	,,	0·56	,,
Chloroform	,,	1·40	,,
Ethyl acetate	,,	1·07	,,
Alcohol	,,	1·52	,,
	Total	4·99	%

In order to glean some idea of the nature of the drug, two portions (500 g. each) were treated respectively with (*a*) sodium carbonate (30 g.) and (*b*) hydrochloric acid (2 %).

(*a*) On distillation in steam, 0·7 g. of an essential oil was obtained which was neutral, and contained no furfuraldehyde. The aqueous distillate was also neutral, thus proving the absence of volatile bases.

(*b*) In this case the acidic solution was filtered from the marc, and although the filtrate gave the usual alkaloidal reactions, only resinous products were obtained.

For the purpose of a complete examination of the drug, a quantity (5 kg.) of the ground material was thoroughly extracted with light petroleum, ether and alcohol.

Examination of the light petroleum extract.

After the removal of the solvent, there remained a dark green viscous mass amounting to 15 g. The residue was taken up in ether when, on keeping, a small quantity of sparingly soluble substance separated as a white amorphous powder. This was collected and washed thoroughly with ether when it was found to melt between 74° and 77°; and, after recrystallisation from ethyl acetate, it was obtained in colourless plates melting at 82° (found, C = 78·05 % and H = 12·7 %). On hydrolysis with alcoholic potash, an alcohol melting at 80° and an acid melting at 77·5–78° were obtained. The above compound is evidently an ester, and in view of the subsequent isolation of arachidic acid, it is very probable that the acidic portion of the ester is also arachidic acid.

The ethereal liquid, after the removal of the above-described ester, was extracted with solutions of ammonium and sodium carbonates, and 5 % sodium hydroxide.

The ammonium carbonate removed a mere trace of substance.

The sodium carbonate removed 0·1 g. of an acid which, when recrystallised from ethyl acetate, melted at 62°. This acid was probably palmitic acid, but the amount obtained was too small to confirm by analysis.

The sodium hydroxide also removed a further small quantity of the acid melting at 62°.

The ethereal liquid, containing the neutral portion of the extract soluble in light petroleum was finally dried over anhydrous sodium sulphate and the solvent removed, when there was left a quantity of viscous product. This was hydrolysed with alcoholic potassium hydroxide, and worked up in the usual manner for the isolation of the unsaponifiable constituents and the combined fatty acids. In this way there were isolated (*a*) a phytosterol (M.P. 134°) which gave an acetyl derivative melting at 117°; (*b*) a *new* unsaturated alcohol (B.P. 145°/25 mm.) agreeing with the formula $C_{26}H_{42}O$ (found: $C = 84{\cdot}6$ %; $H = 11{\cdot}3$ %. Calc. for $C_{26}H_{42}O$: $C = 84{\cdot}8$ %; $H = 11{\cdot}3$ %). This alcohol possesses the characteristic odour of the drug and appears to be the odoriferous principle; it gave the colour reactions of the phytosterols; (*c*) traces of unsaturated fatty acid and (*d*) arachidic acid (M.P. 77°). (Found: $C = 76{\cdot}6$ %; $H - 13{\cdot}0$ %. Calc. for $C_{20}H_{40}O_2$: $C = 76{\cdot}9$; $H - 13{\cdot}0$ %.)

Examination of the ethereal extract.

This was a dark green soft solid amounting to 20 g. It gave only (*a*) a grey amorphous solid (M.P. 86°) and (*b*) a colourless crystalline substance (M.P. 82°), both being insufficient to identify. There was a complete absence of any glucoside in this extract.

Examination of the alcoholic extract.

This extract consisted of a dark coloured resin amounting to 150 g. It was mixed with water, and the mixture distilled in a current of steam. The distillate contained a small amount of volatile oil, but nothing acidic or basic. After the above operation, there remained in the flask an orange-coloured aqueous liquid together with a quantity of dark brown resin. The latter amounted to 40 g., from which nothing definite was isolated.

Examination of the aqueous liquid.

This liquid, after being separated from the resin, was concentrated *in vacuo* to about one litre and repeatedly extracted with ether.

Isolation of scopoletin, $C_{10}H_8O_4$. The ethereal extracts were united and concentrated to small volume, when, on keeping, 7 g. of a pale yellow solid separated. This was collected and recrystallised from alcohol, giving pale yellow needles (M.P. 204°), and a brilliant blue fluorescence with alkalis. The substance was identified with scopoletin. (Found: C = 62·5 %; H = 4·18 %. Calc. for $C_{10}H_8O_4$: C = 62·5 %; H = 4·2 %.)

An *acetyl* derivative was prepared which separated from alcohol in slender colourless needles, M.P. 177°. (Found: C = 61·25 %; H = 4·4 %. Calc. for $C_{10}H_7O_4.COCH_3$: C = 61·5 %; H = 4·3 %.)

After washing the ethereal solution and drying, 0·1 g. of a pale yellow solid separated on keeping for a short time. It was found to be phenolic in character, but it was quite amorphous and had no definite melting point.

Isolation of a basic principle. The original aqueous liquid, after being extracted with ether as described above, gave precipitates with the usual alkaloidal reagents and possessed a bitter taste. A portion of the solution was therefore made alkaline with sodium carbonate and repeatedly extracted with ether. The ethereal extracts were united, dried and the solvent removed, when there remained a small quantity of a gummy mass. The product contained nitrogen and gave reactions for an alkaloid. It could not be obtained crystalline. A solution in hydrochloric acid gave a brown amorphous product with mercuric chloride, and a *picrate* was readily formed which was soluble in hot alcohol, but separated in the amorphous state, melting with decomposition at 125°. No crystalline derivatives could be prepared.

The remainder of the aqueous liquid was concentrated *in vacuo* to a syrup, and a small portion of the latter was treated with phenylhydrazine acetate, when an osazone separated. This was recrystallised from dilute pyridine, when it melted at 210° thus being identified as *d*-phenylglucosazone.

Isolation of a hitherto undescribed glucoside of scopoletin. The concentrated aqueous liquid was mixed with a quantity of absolute alcohol when a precipitate separated. This redissolved on warming and again separated on cooling as a brown solid.

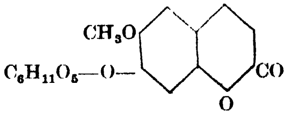
After filtration the substance was recrystallised from dilute alcohol using a little charcoal to remove colouring matter, when it separated in rosettes of needles melting at 226–228°.

The compound gave scopoletin and glucose on hydrolysis with dilute acid, and analysis proved it to be the glucoside of scopoletin having the formula $C_{16}H_{18}O_9, 2H_2O$. Dried at 100°. Found: C = 48·7 %; H = 5·6 %. Calc. for $C_{16}H_{22}O_{11}$: C = 49·2 %; H = 5·6 %. Dried at 130°. Found: C = 51·2 %; H = 5·35 %. Dried at 160°. Found: C = 51·27 %; H = 5·3 %. Calc. for $C_{16}H_{20}O_{10}$: C = 51·6 %; H = 5·3 %.

It was found impossible to remove the second molecule of water, and a separate determination of the substance dried at 160° showed a loss of 4·6, the calculated amount for 1 H_2O being 4·8 %.

The above glucoside has not been described hitherto in the literature and it is proposed to designate it *fabiatrin*.

Fabiatrin is a colourless solid which crystallises in needles, readily soluble in hot water, but very sparingly soluble in the usual organic solvents in the cold. The aqueous solution gives a yellow colour with ammonia and caustic soda, indicating the opening of the pyrone ring, and the constitution of the glucoside may be represented thus:



Isolation of a saponaceous substance. The dilute alcoholic solution of the syrup, after the removal of the glucoside, was concentrated to small volume and kept for some days. A further quantity of fabiatrin separated, the total amount obtained being 7 g. After removal on the filter, the filtrate was digested with a quantity of absolute alcohol, when a brown amorphous solid separated, whose properties indicated that it belonged to the saponins. A very dilute aqueous solution produced a foam which remained stable for many days. It possessed an exceeding bitter taste, but in the dry state was not sternutatory.

PHYSIOLOGICAL TESTS.

These test were kindly conducted by Prof. W. J. Dilling and the results (indicated briefly) show that the drug is poisonous to animals, a decoction having proved fatal to frogs, rabbits and cats. The poisoning effect appears to be due to a saponin, and such a substance has been shown to be present.

A separate examination of scopoletin has shown this substance to be quite inert, both physiologically and bacteriologically—the latter experiments being kindly done by Prof. J. M. Beattie.

The physiological action of fabiatrin will be described in due course.

SUMMARY.

A complete examination of the aerial stems of *Fabiana imbricata* (Ruiz and Pavon) has been conducted, and the following substances have been isolated. A neutral essential oil which gave a negative reaction for furfuraldehyde; an ester (M.P. 77·5–78°): palmitic acid; a phytosterol (M.P. 134°); a new unsaturated *alcohol*, $C_{26}H_{42}O$ (B.P. 145°/25 mm.), possessing the fragrant odour of the drug; traces of unsaturated acids; arachidic acid; scopoletin; a basic compound which gave an amorphous picrate (M.P. 125° with decomposition); a sugar giving *d*-phenylglucosazone; a new glucoside of scopoletin, $C_{16}H_{18}O_9 . 2H_2O$ (M.P. 226–228°), designated *fabiatrin*; and an amorphous substance possessing the properties of a saponin.

In conclusion, the authors express their warmest thanks to Prof. I. M. Heilbron for suggesting the research; to Dr C. J. MacAlister, F.R.C.P., for his instrumentality in providing the material, and also a grant towards the expenses of the investigation; and to Profs. Dilling and Beattie for conducting the physiological and bacteriological tests.

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CXXXV. VARIATIONS IN THE SERUM-CALCIUM OF RABBITS.

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(*Received June 21st, 1927.*)

In a comparison of the effect on rabbits of the parathyroid hormone with that of insulin and various other substances, Davies, Dickens and Dodds [1926] showed that the serum-calcium could be raised by the injection of insulin at regular intervals of time. The curve that they obtained for the serum-calcium resembled those published by Clark and Collip [1926, 2] showing the results of similar repeated injections of parathyroid extract into dogs. Further, by following Clark and Collip's [1926, 1] method of extraction of the parathyroid hormone, they obtained a product from pancreas which, when injected into rabbits, caused an increase in the serum-calcium; but both with this extract and that of the actual parathyroid glands a rise was only obtained when very large doses were used. The authors suggested that the parathyroid hormone, like insulin, might be fairly widely distributed in the body. If these two substances occur together in the pancreas, owing to the marked similarity between their properties they might be extracted together and some of the active principle of the parathyroid would then remain as an impurity in the final product of insulin. Hence, the effect observed on the serum-calcium of the rabbit might be due, not to some additional property of insulin, but to its association with this impurity.

Should this hypothesis prove to be correct, a number of practical results would follow, and it appeared worth testing with regard to the possibility of obtaining (*a*) a new criterion for the purity of insulin—that it should have no effect on the serum-calcium; (*b*) a use for apparently worthless by-products of insulin manufacture; and (*c*) an explanation of the frequent very erratic response of rabbits to insulin. While the hypoglycaemic effect is known to last for only a few hours, that on the calcium might be much more prolonged and might account both for the "bulk variation" of groups of animals in their reaction to insulin, which has been repeatedly observed by workers engaged in its physiological assay, and also for the individual variations of particular animals.

Method.

A group of ten young rabbits was selected for experiment. Four acted as controls throughout, two receiving doses of saline, two having no injections at all.

All doses were calculated on the weight of the animal, and dilutions were made such that a volume of 0·25 cc. per kg. was always injected. The animals were fed throughout unless otherwise stated, their diet consisting of hay, oats and cabbage.

Serum-calcium determinations were carried out by the method of Kramer and Tisdall [1921] modified by Clark and Collip [1926, 3].

To test the method, seven separate estimations of serum-calcium were made on the same blood and gave the following values in mg. per 100 cc. of serum: 15·37, 15·27, 15·18, 15·18, 15·27, 14·88, 15·08. Hence, a difference of 0·5 mg. per 100 cc. can be regarded as outside the range of experimental error.

THE EFFECT OF INSULIN ON THE SERUM-CALCIUM.

Since Davies, Dickens and Dodds observed a significant rise four hours after the injection of what they describe as a sample of "commercial insulin," a number of preliminary experiments were made in which blood samples were taken before and four hours after injection; but neither with the purest nor with very impure samples of insulin was any rise obtained, and both the rabbits receiving insulin and the controls which received none gave similar results.

The following is a typical experiment.

The rabbits were given a dose of one unit per kg. of a very impure preparation of which one unit was contained in 8 mg. They had received their morning ration of cabbage about half an hour before the experiment was started, and hay and oats were present in their cages throughout. The results are shown in Table 1, and, for the sake of comparison, figures are also given for an experiment using the same animals in which precisely similar conditions were observed but no injections were given to any of them.

Table 1.

	(1) Nov. 10th			(2) Nov. 17th		
		Serum-calcium mg. per 100 cc.			Serum-calcium mg. per 100 cc.	
Rabbit no.	Dose	Initial	4 hours	Dose per kg.	Initial	4 hours
N 1	None	14·50	13·82	1 u.	15·09	13·44
N 2	,,	14·31	13·44	,,	14·31	12·96
N 3	,,	17·12	14·60	,,	14·89	13·54
N 4	,,	14·89	13·73	,,	14·41	14·41
N 6	,,	15·57	14·21	,,	14·50	14·02
O 6	,,	16·44	13·85	,,	13·82	12·47
Average		15·47	13·94	—	14·50	13·47
Change from initial...		—	- 1·53	—	—	- 1·03
Control 1. No insulin. Saline.						
O 1	None	13·82	13·35	0·25 cc.	14·01	11·70
O 10	,,	14 79	13·24	,,	13·93	12·86
Average		14·31	13·30	—	13·97	12·28
Change from initial...		—	- 1·01	—	—	- 1·69
Control 2. No insulin. No saline.						
P 9	None	15·09	13·93	None	14·12	13·56
P 10	,,	15·18	13·68	,,	13·73	12·67
Average		15·14	13·81	—	13·93	13·12
Change from initial...		—	- 1·33	—	—	- 0·81

It will be seen that in both cases a fall was obtained. Work by Clark [1920], Moritz [1925] and Stewart and Percival [1927] suggests that this is the usual effect of haemorrhage on rabbits and a number of results obtained in the course of this investigation lead to the same conclusion. Haemoglobin estimations indicate a small but progressive dilution of the blood with respect to this constituent when rabbits are bled at frequent intervals of time. The two observations may possibly be correlated. In the above experiments, however, the animals were fed, and, as will be shown later, this may have a considerable effect: it is possible that with careful control of the diet the rabbit might show the same constancy with regard to the calcium content of its serum as has been demonstrated in the case of the cat (Stewart and Percival).

There appeared to be two possible explanations of these negative results:

(*a*) that the particular insulin samples used would cause a rise in serum-calcium at some other interval of time than that investigated in these preliminary experiments; or

(*b*) that only certain rabbits show this effect.

A. (i) *The effect of repeated small doses of insulin when rabbits are fed with hay, oats and cabbage.*

A number of experiments were performed in which the rabbits were given injections of 0·5 or 1 unit per kg. every two hours for six hours, blood samples being taken either at the 4th or 5th and 20th hours, but in no case was any rise in the serum-calcium obtained. Finally the curve for the whole 24 hours was followed, injections being made every four hours, and the conditions described by Davies, Dickens and Dodds being as far as possible observed.

According to the usual routine of this laboratory the rabbits were given hay, oats and cabbage at 9 a.m. and 5 p.m., and in addition during the night cabbage and oats were given at 3 a.m.

The results are shown in Fig. 1.

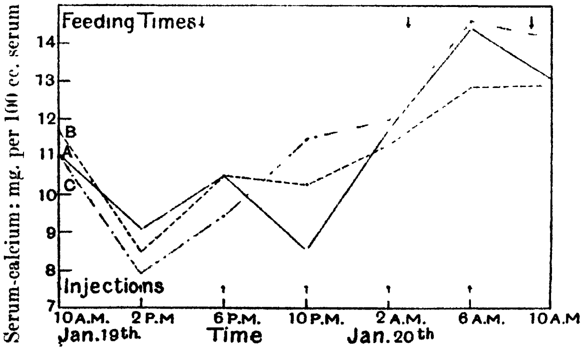

Fig. 1. A. Average curve for six rabbits receiving doses of 0·5 unit of insulin per kg every four hours.

B. Average curve for two rabbits receiving doses of 0·25 cc. per kg. of normal saline solution every four hours.

C. Average curve for two rabbits having no injections.

Both the controls and the rabbits receiving insulin showed a preliminary fall followed by a rise to a high value. This rise cannot be due to insulin because the animals that received none reacted similarly. It is a significant fact that on every occasion on which cabbage was given there was a considerable increase. An apparent exception to this is seen in the rise at 2 a.m., but a probable explanation lies in the fact that the cabbage given at 5 p.m. was not all consumed until much later; thus, it was observed at 10 p.m. that all the rabbits on insulin and one of the controls still had cabbage in their cages left over from the 5 o'clock meal. Unfortunately no note was made of this at the time nor observation made on the other three controls, because the importance of these details of feeding was not then recognised. During the next four hours all the cabbage was eaten, since none remained in the cages at 2 a.m. Throughout the whole period oats and hay were present in all the cages, only the cabbage varied.

One rabbit went into convulsions at 9 o'clock and was given a large quantity of cabbage in the hope that it might recover without the necessity of an injection of glucose. It recovered without sugar and showed the greatest rise in serum-calcium.

A. (ii) *The effect of repeated small doses of insulin when rabbits are fed with hay and oats only.*

Fig. 2 shows the results of four-hourly doses of 0·5 unit per kg. of the same sample of insulin as was used in the above experiment, the rabbits receiving their usual diet but with the cabbage omitted.

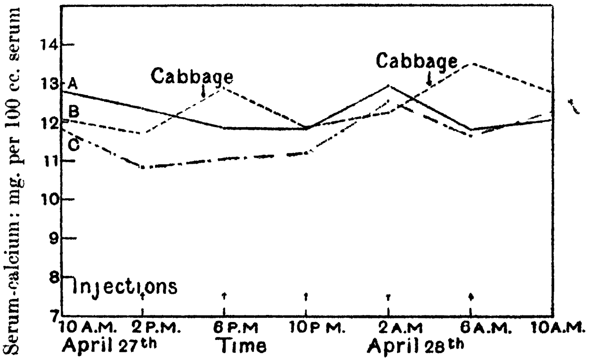

Fig 2. A. Average curve for six rabbits receiving doses of 0·5 unit of insulin per kg. every four hours when cabbage was withheld.

B. Average curve for two rabbits receiving no injections but to whom cabbage was given twice during the experiment.

C. Average curve for two rabbits who received no insulin and no cabbage.

Cabbage was withheld from 12 noon on the day preceding experiment and water given instead until 5 o'clock in the evening in order to avoid any possible complications from undue thirst, cabbage being normally the chief source from which these animals obtain water.

Of the four controls which received no insulin, two were given cabbage twice during the course of the experiment, at 5 p.m. and at 4 a.m. Both showed a rise in the serum-calcium at 6 p.m. and 6 a.m. It will be seen that the largest deviations from the initial values are shown by these two and that no changes in the serum-calcium of the animals that were injected can be ascribed to their doses of insulin.

B. *The effect of insulin convulsions.*

It is noticeable in the experiment reported by Davies, Dickens and Dodds in which they gave about 0·5 unit per kg. of insulin that the rabbit went into convulsions twice during the 24 hours in spite of the fact that it was fed throughout on cabbage and bran-mash. This led to the consideration that possibly only very sensitive rabbits show a rise in serum-calcium following insulin injection, and that actual convulsions might even be the cause. Hence, three very sensitive rabbits were selected which had been used for some time for insulin testing and were known to convulse on a dose of 0·5 unit per kg. when starved, and a series of experiments was performed with these animals (Table II). Again the results point to the conclusion that when a rise was observed it was caused by cabbage.

Table II.

Date	Conditions of experiment	Dose of insulin in units per kg.	Serum calcium. Average change in 4 hrs after injection, in mg. per 100 cc.
Nov. 3, 1926	Fed at 9 a.m. with oats, hay and cabbage. Injected at 10 a.m.	0·5	−1·11
Nov. 8	Fed at 9 a.m. with oats, hay and cabbage. Injected at 10 a.m.	1·0	−0·56
Nov. 24	Starved for 22 hours. Injected at 10 a.m. All convulsed within 2 hours and were given sugar and cabbage	1·0	+1·32
Dec. 1	Starved for 22 hours. Injected at 10 a.m. All convulsed within 2 hours and were left in convulsive state for 1½ hours before being given sugar and cabbage	1·0	+1·26
Dec. 8	Starved for 22 hours. Injected at 10 a.m. and fed with hay, oats and cabbage at 10.30 a.m. in order to avoid convulsions	1·0	+1·10
Dec. 15	Ditto. (N.B. One convulsed in spite of feeding and was given sugar)	2·0	+1·26
Dec. 29	Starved for 22 hours. Initial blood samples taken at 10 a.m. Given oats, hay and cabbage at 10.30 a.m.	None	+1·26
Jan. 5, 1927	Starved for 22 hours and throughout experiment	None	−0·03
Jan. 12	Starved for 22 hours. Initial blood samples taken at 10 a.m. 6 cc. 20 % glucose injected subcutaneously at 1 p.m.	None	+0·52

0·5 and 1 unit per kg. failed to effect any increase in serum-calcium or to produce convulsions when the rabbits were fed. After starvation for 22 hours (according to the usual routine for insulin testing) a rise was obtained with 1 unit per kg., both after convulsions when sugar and cabbage were given and also when convulsions were avoided by feeding the animals with hay, oats and cabbage half an hour after injection. The rise obtained could not be increased by withholding food and leaving the animals in convulsions for an hour and a half nor by doubling the dose. A control experiment in which the rabbits were fed with hay, oats and cabbage half an hour after the initial blood sampling but were given no injections confirmed the suspicion that insulin was not the cause of the rise obtained in the earlier experiments.

It is a routine in this laboratory that when glucose is used as an antidote to insulin convulsions the rabbit is also given a few cabbage leaves, the consumption of which is taken as a sign of recovery. Thus, on every occasion when the injection of sugar was necessary, the animals were also fed with cabbage.

An examination of the results shows that a rise was obtained after starvation in the following circumstances:

1. On feeding with cabbage and oats.
2. On feeding with cabbage and oats after the injection of insulin.
3. On feeding with cabbage and injecting sugar after the onset of insulin convulsions. (Sugar itself has not been shown to have any effect on the serum-calcium, either by us or by Davies, Dickens and Dodds.)

The only factor that did not vary is the consumption of cabbage. On no occasion was a significant rise obtained when cabbage was not given.

THE EFFECT OF CABBAGE ON THE SERUM-CALCIUM.

The conclusion having been reached by two separate series of experiments that any rise in serum-calcium following insulin injection is caused by cabbage and not by the insulin, the effect of cabbage alone was then tested.

The following procedure was followed.

At 12 noon on the day preceding experiment cabbage was removed from the cages and the animals were given water until 5 p.m. In order to control any possible effect of the water two of the controls were given none. Samples of blood were taken at 10 a.m., and six rabbits were then given a plentiful supply of cabbage which was maintained for 2 hours and then removed. The last four rabbits as usual served as controls and received no cabbage. All were kept supplied throughout with oats and hay, and serum-calcium determinations were made at two-hourly intervals. The results are shown in Fig. 3.

The only rabbit receiving cabbage which failed to show an increased serum-calcium ate none (*N* 6). For the purpose of averages it is therefore included among the controls. Those animals that were given no cabbage showed a slight fall.

Table III.

Exp 1.	Feb. 9th. Cabbage at 10 a.m. Serum-calcium mg. per 100 cc.				*Exp* 3.	April 6th. Cabbage at 11.30 a.m. Serum-calcium mg. per 100 cc.			
Rabbit no.	10 a.m.	12 noon	2 p.m.	4 p.m.	Rabbit no.	9.30 a.m.	11.30 a.m.	1.30 p.m.	3.30 p.m.
N 1	13·44	15·38	14·31	14·12	*U* 1	13·24	13·04	15·53	14·64
N 2	13·93	15·38	14·03	13·05	*U* 2	14·34	13·14	15·24	14·24
N 3	14·03	14·79	13·93	14·31	*U* 3	14·44	13·84	15·93	14·34
N 4	13·54	14·31	13·35	13·24	*U* 4	14·64	13·94	15·24	13·94
**N* 6	(13·24	12·76	12·76	12·38)	*U* 5	13·64	11·05	14·94	13·54
O 6	11·99	12·57	12·09	10·47	*U* 6	14·44	13·84	15·14	14·04
Average	13·39	14·49	13·54	13·04		14·12	13·14	15·34	14·12
Change from initial		+1·10	+0·15	−0·35		—	−0·98	+1·22	—
Control 1. No cabbage. Water given on the day preceding experiment.									
O 1	12·57	12·38	11·70	11·21	*U* 7	13·04	12·94	13·04	12·05
O 10	12·47	12·47	11·99	11·90	*U* 8	13·54	12·15	13·04	12·35
Average including *N* 6	12·76	12·54	12·15	11·83		13·29	12·55	13·04	12·20
Change from initial		−0·22	−0·61	−0·93		—	−0·74	−0·25	−1·09
Control 2. No cabbage. No water.									
P 9	13·15	12·86	12·18	11·12	*U* 9	14·24	13·84	12·84	13·04
P 10	12·47	13·35	11·99	11·21	*U* 10	13·14	13·14	11·65	11·55
Average	12·81	13·11	12·09	11·17		13·67	13·49	12·25	12·30
Change from initial		+0·30	−0·72	−1·64		—	−0·20	−1·44	−1·39

* *N* 6 ate no cabbage and is therefore omitted from the average and included in Control 1.

This experiment (Exp. 1, Table III) appeared to prove definitely that the previous conclusion as to the effect of cabbage was correct, and further confirmation was obtained by an experiment on the three abnormally sensitive rabbits which gave the same result.

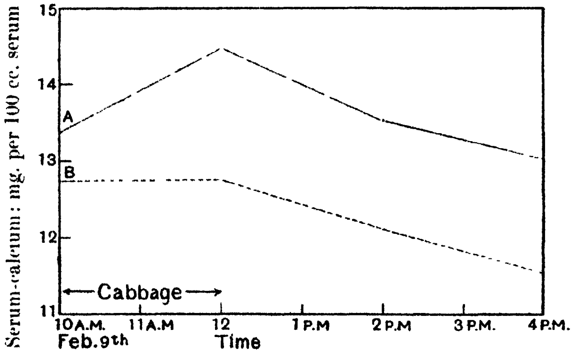

Fig. 3. A. Average curve for five rabbits fed with cabbage between 10 a.m. and 12 noon.
B. Average curve for five rabbits who were given no cabbage.

The next step attempted was to determine what constituent of cabbage has the property of raising the serum-calcium. For the purpose of making a systematic investigation of this problem it was thought advisable to start off with an entirely new set of rabbits. As a preliminary experiment, therefore, the above test was repeated on these young rabbits. The results were entirely negative, the experimental animals reacting in the same way as the controls.

It is a well-known fact reported by many different observers that when the serum-calcium is high it is not easily changed, whereas when it is low it may be increased by a variety of means. Also it has been stated by Grant and Gates [1924], and confirmed by this work, that rabbits show a seasonal variation in serum-calcium, giving minimum values in January and high values in May and November. This is shown in Fig. 4, which gives a curve for the average of the initial values for ten rabbits during 3 months. Hence, the possible explanation of the different response to cabbage of the young rabbits tested in the middle of March from that of the group tested 5 weeks earlier is that the new ones were very near their maximum at the beginning of the experiment. Therefore, when it was repeated again on the same young animals 3 weeks later, instead of cabbage being given immediately after the initial blood samples were taken, it was given 2 hours later when the calcium content of the serum had already fallen. This time all the rabbits receiving cabbage showed a considerable rise (Fig. 5). The results are shown in Exp. 3, Table III.

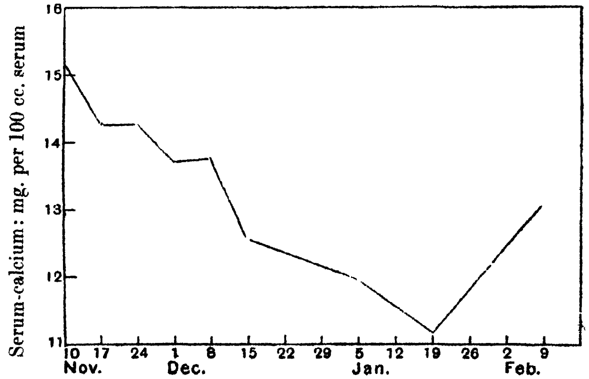

Fig. 4. Curve showing the average "seasonal variation" of the initial serum-calcium values for ten rabbits over a period of three months.

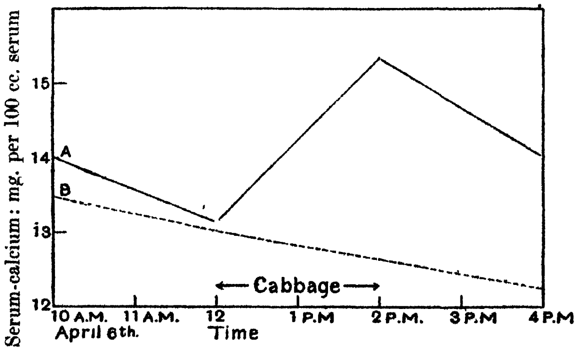

Fig. 5. A. Average curve for six rabbits fed with cabbage between 12 and 2 p.m. B. Average curve for four rabbits who were given no cabbage.

CONCLUSIONS.

1. Insulin causes no rise in the serum-calcium of rabbits.
2. Insulin convulsions cause no rise in the serum-calcium of rabbits.
3. An immediate rise in the serum-calcium of rabbits occurs on feeding with cabbage, except when the initial value may be regarded as exceptionally high. The cause of this effect remains to be found.

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CXXXVI. STRUCTURE AND ENZYME REACTIONS. PARTS I AND II.

THE SYSTEMS UREA-UREASE-CHARCOAL AND POLYSACCHARIDE-AMYLASE-CHARCOAL.

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The question of the applicability of the law of mass action to reactions in the living organism has long been considered as one of the most important problems in the dynamics of life phenomena.

The chief difficulty in the solution of this problem is that these reactions take place in a heterogeneous medium, in which, apart from purely chemical reactions, we encounter processes of diffusion, and the whole question is further complicated by the fact that most of the reacting substances are in the colloidal state.

For this reason, all except ionic reactions and those connected with the last stages of disintegration or the first stages of synthesis, are reactions involving all those factors connected with reactions of hydrophile colloids in heterogeneous media, *i.e.* reactions of adsorption, and in general, all surface phenomena, to which the law of mass action is, at least in the state of presen day knowledge, inapplicable.

In this paper we shall consider the questions of the applicability of the law of mass action to enzyme reactions. In general, in so far as the kinetics of enzyme reactions in homogeneous systems are concerned, if we can, in general, consider any enzyme system to be homogeneous, these conform to the law of mass action, as has been pointed out by Michaelis and Menten [1913] and Josephson [1925].

It is a question whether the same holds good for enzyme reactions *in vivo*, and whether reversible enzyme reactions may here be interpreted on the basis of the concentration of the substrates. Here we encounter a whole series of incomprehensible facts, apparently irreconcilable with the law of mass action, a classical example of this being the different behaviour of amylase *in vitro* and *in vivo*.

Some factor must therefore exist in the structure of the cell which alters the course of reactions and their final equilibrium point. This factor need not necessarily be connected either with the nervous or the endocrine system, but may be connected with the actual structure of the liver cell. A whole series of examples could be cited, showing that this phenomenon is not limited to amylase, but may also be encountered among the most varied reactions of enzymic hydrolysis, involving colloidal constituents, such as proteins, carbohydrates and nucleic acids.

Apart from the question of the direction of the reaction and its final equilibrium point, it is a point of considerable interest that the velocity of enzyme reactions depends to a large extent upon the structural conditions of the environment in which the enzyme acts. In general, enzymic and vital processes may be classified into those in which the velocity of reaction is accelerated by the destruction of structure, and those in which it is retarded, most hydrolytic ferments belonging to the former class.

Hofmeister [1901], on the basis of Bütschli's theory of the alveolar structure of protoplasm, considers that reactions take place in the more liquid portions of the cell. Thus ferments, to which the colloidal parts of the partitions are impermeable, cannot liberate themselves from the latter, in contrast with those crystalloids, taking part in the reactions of the cell, which are able freely to circulate.

Hofmeister did not analyse more closely the possible causes of the binding of the enzymes. These may be extremely heterogeneous. Hedin [1915] was one of the first to draw attention to the phenomenon of adsorption as a possible factor in the explanation of variability of the velocity of enzyme reactions *in vivo*.

Lesser [1920] showed that the retardation of the decomposition of glycogen may take place, not only *in vivo*, but may also be reproduced experimentally by the addition of some adsorbent to the solution. Lesser interpreted his results in the following way. Only that portion of ferment which is in solution is active, the adsorbed portion being inactive. Substances which lower surface tension increase the active concentration of ferment by elution of the latter. This effect is similar to the hypoglycaemia produced by alcohol and other narcotics *in vivo*.

This phenomenon has been the subject of numerous researches, based upon the assumption that adsorbed enzymes are inactive. Certain facts tending to discredit this assumption may, however, be found, such as Meyerhof's [1914] discovery that saccharase, adsorbed on colloidal ferric hydroxide, retains its enzymic activity. Admitting therefore, that adsorption need not necessarily deprive enzymes of their activity, the interpretation of Lesser's experiments becomes exceedingly difficult.

The possibility may arise that, if enzymes lose their activity not through adsorption, but in accordance with Hofmeister's [1901] conception, at least in certain cases, changes in the velocity of enzyme reaction in heterogeneous systems may be due to the separation of the ferment from its substrate. In that case, we should have to admit that this retarding effect would arise when both the enzyme and its substrate are adsorbed, and this appears to be the case for the system amylase-glycogen.

Certain unpublished experiments carried out by one of us [cf. Przylecki, 1922], in 1918-1920, also support this interpretation. In these experiments we found that, during the months in which Lesser found almost complete absence of glycogenolytic action in the frog's liver, *i.e.* from November to January, the liver synthesises glycogen quite efficiently from glucose. Thus frogs placed in 7 % glucose at 15° exhibited a considerable increment of glycogen, the content of the latter in the liver increasing on the average by 40 % over a period of two weeks. Almost identical results were obtained in experiments in which the glycogen content of the liver was determined at the beginning of the experiment, using in this case summer frogs at the same temperature (see Table I).

Table I. *Glycogen content of lobes of the livers of frogs kept* 10 *days in a* 7 % *solution of glucose at* 15°.

		Content in the analysed lobe at beginning of experiment in % live weight		Same after ten days	
Month	No. of experiments	Average	Deviations	Average	Deviations
December	5	5·5	4·8–6·0	7·8	7·1–8·3
June-July	5	4·8	3·9–5·9	6·8	5·3–7·9

If therefore, Lesser's hypothesis were correct, it could be reconciled with our findings only on the assumption that the synthesis of glycogen depends upon a different enzyme from amylase, since any other interpretation would necessarily involve accepting the view that a free ferment acts as efficiently as when adsorbed. The difference in the velocity of hydrolysis of glycogen between summer and winter livers would not, in that case, be due to differences in the amount of amylase present, but to the distance separating the enzyme from its substract. Should this be so, it would be clear that the enzyme acts equally efficiently for the synthesis as for the hydrolysis of glycogen, and further, the reason for the existence of seasonal differences in the velocity of the latter process would be explained, since the enzyme and substrate may be more or less separated from one another, according to the season.

In order to decide which of the above views is correct, *i.e.* to prove that the velocity of enzyme reactions is affected, not by adsorption of the enzyme, but by spatial separation of the latter from the substrate, a whole series of experiments would be necessary.

The experimental part of this research has as its object the verification of the following postulates upon which the above reasoning is based.

1. That adsorbed enzymes retain their activity; *i.e.* that the velocity of enzyme reactions, in systems where the enzyme is strongly adsorbed on to

some adsorbent, and the substrate slightly or not at all, is equal to or only slightly less than in the absence of the adsorbent.

2. That, in systems containing an adsorbent, the velocity of enzyme reactions depends upon the concentration of unadsorbed substrate.

Experiments conducted upon the system urease-charcoal-urea confirmed the first postulate, showing that the velocity of decomposition of urea is independent of the presence of an adsorbent, in spite of the fact that 90 % of the urease is adsorbed. Urea causes scarcely any elution of the ferment and the addition of alcohol has little influence upon the velocity of decomposition of urea.

We may hence conclude that in those conditions where an adsorbed enzyme suffers no loss of catalytic power, and the substrate is not adsorbed, the change from a homogeneous to a heterogeneous system, due to the introduction of the adsorbent. does not influence the direction of the reaction, its final equilibrium point, or the velocity of enzymic reaction.

We shall next proceed to examine those systems in which both the enzyme and the substrate undergo adsorption. Such a case is afforded by the systems amylase-dextrin or glycogen-charcoal. Here we find that dextrin and glycogen are strongly adsorbed by charcoal, that the addition of such substances as butyl and propyl alcohols, ether and chloroform greatly diminishes the degree of the adsorption, that amylase is very strongly adsorbed by charcoal and that the addition of alcohols, chloroform or ether. in such quantities as diminish the degree of adsorption of dextrin or glycogen by 50 % does not produce this effect upon amylase. Since the completion of these experiments we have found a confirmation of this fact in a paper published by Unna [1926].

Experiments carried out with the object of comparing the velocity of enzymic hydrolysis in the presence or absence of charcoal show that this velocity increases inversely as the quantity of added adsorbent. In this way, the quantity of glycogen hydrolysed by amylase in the presence of charcoal may be reduced to 5 % of that which would normally be decomposed.

Alcohols added to such systems in such quantities as cause no elution of adsorbed amylase, but only of glycogen or dextrin, cause a commensurate increase in the velocity of hydrolysis of these polysaccharides.

The above results show that, in those cases examined, the cause of differences in the velocity of enzyme reactions lies in adsorption, not of the enzyme, but of the substrate. It would therefore appear that adsorbed enzymes act quite efficiently, and in order to show that this is the case we have been able, making use of the fact that the adsorbed ferment does not undergo elution on treatment with water or glycogen solution, to prove that amylase completely adsorbed on charcoal is capable of hydrolysing glycogen or dextrin.

The next point which we wished to establish was that an adsorbed ferment is as active as when not adsorbed. This would also show that differences in reaction velocity depend, not upon adsorption of ferment, but of substrate. That such is the case was shown by the fact that the velocity of hydrolysis of glycogen or dextrin in the presence of charcoal depends upon the concentration of unadsorbed substrate, and that where the same quantity of ferment was taken and the concentration of free substrate was the same, the presence of charcoal made no difference to the velocity.

From the results obtained for the system amylase-polysaccharide-charcoal may be drawn the following conclusions. Firstly, adsorbed or free amylase possesses the same activity. Secondly, differences in the velocity of hydrolysis of polysaccharides in the presence or absence of charcoal are due solely to the above described spatial separation of the enzyme from its substrate. This would mean that, whether the enzyme combines chemically with the substrate or whether, as Bayliss [1924, 1925] assumes, an adsorption compound is formed. an adsorbed enzyme is only exceptionally able to enter into contact with an adsorbed substrate. Our figures show that, under these conditions, combination of enzyme with substrate is very limited, if the solution is not stirred, and even with stirring only a minimal combination takes place between the adsorbed enzyme and polysaccharide.

A further extremely important conclusion may be drawn from our experiments, namely, that the law of mass action applies also to enzyme reactions in heterogeneous systems. Up to the present, the equation used to express this law had included the following factors: quantity of enzyme, of substrate and of water. If absolute values be applied in this equation, it is obvious that fundamental differences will exist between ferment systems in the presence or absence of an adsorbent. On the other hand, this need not be the case if not the absolute quantities of the above three components be taken into consideration, but only that part of them which is free to take part in the reaction. In this case, we have shown that the whole of the enzyme present is reactive, whilst, in those experiments in which the whole of the enzyme is adsorbed only the free unadsorbed portion of the substrate is at liberty to enter into chemical or adsorptive combination with it. Hence only this portion takes part in the reaction, and should be exclusively considered in the equation. Introducing this correction, we find that the law of mass action is here followed, and that the velocity of reaction between the whole of the enzyme present and the unadsorbed part of the substrate is independent of the heterogeneity of the system.

Conditions are somewhat more complicated if part of the enzyme is unadsorbed. In this case the possibility would arise that this unadsorbed portion would be free to combine with adsorbed substrate. If as above, we assume that only the unadsorbed fraction of the substrate is free to enter into reaction, and accept this as the active concentration, higher values would be obtained than the theoretical.

Thus we see that an examination of the systems urease-urea-charcoal and amylase-polysaccharide-charcoal yields concurrent results, fully in conformity with our theoretical reasoning. The spatial separation of an enzyme from its substrate by the adsorption of both is a factor inhibitive of enzyme action in heterogeneous systems.

It would be of interest to consider firstly, how general is this phenomenon of the influence of structure, and secondly, whether from the above experiments any other conclusions as to influence of structure on enzyme reactions might be drawn. As to the former problem, Meyerhof and Willstätter and his colleagues [1922, 1925] have shown that adsorbed ferments may act quite normally, without any difference in the degree of their activity.

Hedin's work, however, shows that this fact cannot be generalised, since he found that trypsin and rennin lose their activity on adsorption. We would in this connection emphasise that Willstätter, Waldschmidt-Leitz and Kraut found extremely different degrees of adsorption for the pro- and co-enzymes of trypsin, and it may be that their separation and fixation on the adsorbent leads to the inhibition of their action.

This different behaviour of various enzymes, or even of the same enzyme, using various adsorbents, is probably due to differences in the place of attachment of the enzyme to the adsorbent. Thus it is conceivable that in one case, the adsorbent may be bound to a grouping belonging to the accessory non-characteristic part of the enzyme, whilst in a different case, a group indispensable to the action of the ferment is bound.

Such phenomena as changes in velocity of reaction may, within the living organism, depend not exclusively upon sorption in the most general sense of the word, but may equally well be due to chemical combinations, leading to the local fixation of the enzyme or substrate. In this case, glycogen may enter into some loose combination with some other organic colloid.

Certain analogous phenomena may be produced by mechanical occlusion and precipitation during the passage of some colloid from sol to gel, as exemplified in certain of Henri's [1906] experiments. An enzyme reaction which takes place in a solution of gelatin is inhibited by the presence of gelatin gel.

A certain rôle may also be played here by the degree and condition of saturation of the substrate. Thus, owing to changes in the state of solution of difficultly soluble substances, which readily tend to reprecipitate, their active concentration may vary in spite of the fact that their total concentration remains constant. A number of further possibilities may also exist. Thus certain factors may exist which lead to elution of the enzyme but not of the substrate. Such cases have not so far been noted in the literature of the subject, but are none the less possible. Their effect would be to augment velocity of reaction, since the unadsorbed enzyme would be enabled to react with the substrate.

If we accept the above reservations, and substitute for adsorptive influences all factors tending spatially to separate the enzyme from its substrate, many of the characteristics peculiar to the phenomena of the living cell would be explicable.

A further important conclusion follows from this conception of spatial separation as an inhibitive factor in enzyme reaction. Accepting that in homogeneous systems the total concentrations of enzyme and substrate enter into the equation expressing the equilibrium constant, whilst in heterogeneous systems, in which the enzyme is almost completely adsorbed, only the concentration of unadsorbed substrate enters, then, for reversibly acting enzymes, the final equilibrium point must be different in these two systems, wherever the synthesised body and its disintegration products are differentially adsorbed.

Let us imagine a reversible reaction in a homogeneous system, where. at the equilibrium point the concentration of synthesised product to disintegration products is as 40 : 60. If the former body is more strongly adsorbed than are the latter, then the presence of an adsorbent would shift the equilibrium point in the direction of synthesis, since in the equilibrium equation would figure concentrations of unadsorbed synthesised product and disintegration products. Since the latter are, in the hypothetical case here considered, less strongly adsorbed than are the former, the proportion of the synthesised product in solution would from the commencement be less than that required for equilibrium, and would remain so throughout the experiment. Equilibrium would supervene when considerably more than 40 % of synthesised substance is present: this increment would be larger, the greater the difference in the degree of adsorption of the substrates. Thus a much greater amount of the body should be synthesised in a heterogeneous than in a homogeneous system, and, on the contrary, a much smaller proportion of the synthesised substance should suffer decomposition under the same conditions. Experiments are now in progress with the object of verifying this hypothesis; these are not yet completed, but we consider that they will in all probability confirm our views.

Should this be the case, this would to a certain extent explain why in intracellular conditions the equilibrium point of enzymic hydrolyses lies on the side of synthesis. In such cases as in the synthesis of polysaccharides or proteins, the end-products of reaction are strongly adsorbed, whilst their progenitors or decomposition products are considerably less so.

EXPERIMENTAL.

Solutions of urea and of urease, or of both of these substances together, were added to charcoal, equal proportions of the two former substances taken separately without charcoal, and the amounts of urea converted in both cases were compared. The solutions were filtered either at the beginning of the experiments or after certain intervals of time. The results shown (Tables II to V) are derived from experiments in which both series were carried out simultaneously, using charcoal taken from the same stock. The urease taken was urease powder, purchased from Squibb, a fresh solution being prepared for each series of determinations. In this way, the substances used in each series, both as regards enzyme and adsorbent, were as far as possible uniform.

Urea-ammonia was determined by Schlösing's or Folin's method at a point at which not more than 15 % of the urea present was decomposed. The quantity of urea adsorbed on the charcoal was taken as the difference between that added and that found in the filtrate.

Table II. *Percentage adsorption of urea measured in 2 cc. of solution.*

Amount charcoal added g.	No. of experiments	% urea	% of original urea adsorbed	
			Average	Deviations
0·2	4	0·2	2·4	2·2-2·8
0·4	4	0·4	4·6	3·9–4·8

Table III. *Mg. urea decomposed in the filtrate. Average of five experiments.*

Urease without charcoal	Urease with charcoal	Urease with charcoal and butyl alcohol	Urease with charcoal and propyl alcohol
20·0	0·1	0·1	0·3
19·2	0·3	0·2	0·2

Table IV. *Mg. of urea decomposed.*

Urea, charcoal and urease left in contact without filtration		Urea, charcoal and urease with filtration		Urease and urea		Urease and charcoal filtered and urea added	
0·2 % urea	0·4 % urea	0·2 % urea	0·4 % urea	0·2 % urea	0·4 % urea	0·2 % urea	0·4 % urea
11·8	19·9	3·5	4·8	11·9	20·2	0·0	1·5
11·6	19·8	3·3	5·1	11·5	19·9	0·9	1·0

Table V. *Mg. of urea decomposed.*

Series	Time (hours)		0·1 g. urease, 0·5 g. charcoal and 20 cc. 0·2 % urea					Urease + 0·2 % urea				
			I	II	III	IV	V	I	II	III	IV	V
α	2	No alcohol	5·3	5·5	6·6	5·4	2·7	5·1	5·0	—	5·6	2·8
		Propyl alcohol	—	—	—	—	—	5·1	5·1	—	—	2·8
		Butyl alcohol	—	—	—	—	—	5·0	5·3	—	—	2·7
	4	No alcohol	10·4	10·8	13·1	10·5	5·5	10·2	10·2	13·5	10·7	5·7
		Propyl alcohol	—	—	14·9	11·0	5·7	14·0	10·4	13·5	10·9	5·6
		Butyl alcohol	—	—	15·1	10·8	5·4	—	10·1	13·5	10·7	5·4
β	2	No alcohol	5·2	5·6	6·1	5·7	2·8	5·1	5·2	—	5·8	2·4
		Propyl alcohol	5·3	5·7	—	5·9	2·8	5·2	5·3	—	—	2·3
		Butyl alcohol	5·3	5·4	—	5·9	2·9	5·1	5·3	—	—	2·4
	4	No alcohol	10·5	10·4	12·1	10·7	5·7	10·1	—	11·8	10·8	5·0
		Propyl alcohol	10·7	—	13·9	11·0	5·8	10·2	—	11·8	11·0	5·0
		Butyl alcohol	10·6	—	14·1	11·0	5·8	10·2	—	11·5	10·9	4·9

Series	Time (hours)		0·1 g. urease, 0·5 g. charcoal and 20 cc. 0·4 % urea					Urease + 0·4 % urea				
α	2	No alcohol	10·6	11·4	—	11·7		10·2	10·5	—	11·1	5·9
		Propyl alcohol	11·0	11·5	—	11·7	—	10·3	11·2	—	11·3	5·8
		Butyl alcohol	10·8	11·4	—	11·9	—	10·6	—	—	11·1	5·8
	4	No alcohol	21·1	22·7	—	21·2	—	21·0	20·3	—	21·1	11·9
		Propyl alcohol	21·4	23·0	—	21·4	—	20·3	—	—	21·4	12·0
		Butyl alcohol	21·5	22·8	—	21·4	—	22·5	—	—	21·0	12·0
β	2	No alcohol	10·4	—	—	11·6	—	10·6	11·0	—	11·4	5·6
		Propyl alcohol	10·7	—	—	11·8	—	—	—	—	11·2	—
		Butyl alcohol	10·7	—	—	11·7	—	—	—	—	11·6	—
	4	No alcohol	20·7	22·0	—	21·4	11·2	20·9	21·6	—	22·1	11·3
		Propyl alcohol	—	22·1	—	22·0	—	21·0	22·5	—	22·1	11·3
		Butyl alcohol	—	22·0	—	21·8	—	20·8	21·8	—	22·3	11·2

The second type of experiments was carried out on amylase and dextrin or glycogen. The enzyme used was purified amylase, purchased from Merck;

in some cases the ferment contained in frog's blood was taken, proceeding according to Lesser [1924]. Here too, determinations were made at such intervals that up to 15 % of the polysaccharides taken had been decomposed. The degree of hydrolysis of dextrin was estimated by Bertrand's method. The amount of polysaccharides adsorbed by charcoal was determined by the hydrolysis of the filtrate.

A. *The system urease-urea-charcoal.*

1. *Adsorption of urea.* The values shown in Table II indicate that the specimen of charcoal used adsorbs urea very feebly.

2. *Adsorption of urease.* 0·5 g. of charcoal was added to solutions containing 0·1 g. of urease, the temperature of the solutions being 20°. After 15-30 minutes, the solutions were filtered, the residue was washed with two 5 cc. portions of water, and 0·2 g. of urea added to the filtrate, so that the latter is a 0·4 % solution of urea. Simultaneously an equal quantity of urea was added to a second, also filtered, solution of urease, without previous charcoal treatment. The results obtained, given in Table III, show that in these experiments 85-95 % of the enzyme present undergoes adsorption by charcoal.

The adsorption of urease in the presence of butyl and propyl alcohols was next studied. The results obtained show that neither of these alcohols is able either to cause elution of adsorbed urease, or to diminish the degree of adsorption of the latter when added simultaneously with it (Table III).

3. *Activity of adsorbed urease.* The degree of adsorption of urease was found to be unaffected by the subsequent addition of urea. This fact is of great importance for demonstrating that adsorbed urease retains its enzymic properties, and that the conditions under which free urease in a homogeneous system and adsorbed urease in a heterogeneous system are examined are strictly comparable.

1 cc. of 0·2 % urease solution was added to 10 cc. water containing 0·2–0·5 g. of charcoal. After 30 60 minutes, half of the solution was filtered and urea solution added to both portions, the second portion being also filtered after from 5 to 10 minutes. The amount of unadsorbed urease was estimated by determining the amount of urea decomposed in the first filtrate within two hours. Since the concentration of urea was the same in all cases, differences in the final concentration could arise only from different urease contents. The values given in Table IV show a certain difference between those experiments in which urease was added before and after filtration. These discrepancies are probably largely due to the urea being in contact with the enzyme before filtration. A further cause of these differences lies, however, in the slight elution of urease by urea, as is shown by those experiments in which the quantity of ammonia formed in two hours by filtered urease solutions to which urea is added, is compared with the differences between the amount of ammonia formed in two hours after filtration of a similar solution containing urea, and that present immediately after filtration.

Having thus shown the degree of adsorption of urease by charcoal to be extremely high, and that urea has scarcely any elutive effect, it became possible to investigate the question of the efficiency of adsorbed urease for the reaction of the decomposition of urea.

The velocities of decomposition of urea in the system urease-urea-charcoal, with and without shaking, were compared, and found to be identical. Hence, it would follow that the efficiency of action of urease is independent of its distribution throughout the solution.

B. *The system polysaccharide-amylase-charcoal.*

1. *Adsorption of polysaccharide.* 0·2 or 0·4 g. of charcoal was added to 20 cc. of a solution of dextrin or glycogen, which was filtered after a lapse of 30 minutes. Table VI shows that, in those cases where 0·2 g. of charcoal was added, on the average 50·5 % of the dextrin was adsorbed, whilst with 0·4 g. of charcoal the degree of adsorption was considerably greater, on the average 72·8 %. Glycogen is adsorbed to a slightly greater extent, the average adsorption in a solution containing 0·4 g. of charcoal being 74 %.

The adsorption of the above substrates is therefore quite considerable, as only about 20 % of the given polysaccharide can exist in solution. Using smaller concentrations of substrate, even greater differences between the total content of the latter and its concentration in solution were obtained.

Table VI. *Mg. reducing sugars found after hydrolysis.*

Compound	No. of experiments	In absence of charcoal	0·2 g. charcoal	0·4 g. charcoal
Dextrin	7	Average 13·5 Deviations 13·1–14·0	Average 6·7 Deviations 6·3–7·2	Average 3·7 Deviations 3·3–4·6
Glycogen	4	Average 14·1 Deviations 14·1–14·2	— —	Average 3·4 Deviations 3·2–3·8

Table VII. *Mg. reducing sugars found after hydrolysis. Averages.*

Compound	No. of experiments	Without charcoal	With charcoal		With charcoal and alcohols added		Alcohol added
			0·2 g.	0·4 g.	0·2 g.	0·4 g.	
Dextrin	12	12·2	6·9	3·2	8·5	4·7	Butyl; propyl
Glycogen	4	14·2	—	3·6	—	4·5–4·9	Butyl; propyl

Table VIII. *Mg. reducing sugars found after hydrolysis. Average figures for six experiments.*

Without charcoal	0·4 g. charcoal added	0·4 g. charcoal and various other substances	Other substances added
12·8	3·3	7·0	Amyl alcohol
12·0	2·9	7·6	Chloroform
12·6	3·4	8·2	Ether

In the presence of bodies strongly adsorbed by charcoal, such as propyl or butyl alcohol, considerable elution of glycogen or dextrin took place (see

Table VII). The behaviour of systems containing glycogen is analogous, as may be seen from the results of two experiments conducted on this system, and probably all polysaccharides behave in this way.

The action of amyl alcohol, of ether and of chloroform is, as we see from Table VIII, similar to that of the above alcohols. Thus it appears that more strongly adsorbed substances, such as narcotics, lead to the elution of adsorbed polysaccharides. By making use of this important fact we can change at will the concentration of dissolved polysaccharide without affecting the total amount present.

In all probability, the above observation is of considerable significance in physiological reactions. A second perhaps equally important conclusion which may be drawn from this observation is that the degree of elution of polysaccharides by alcohols is proportional to the degree of adsorption of the former.

2. *Adsorption of amylase by charcoal.* The enzyme-adsorbent system was filtered, a solution of dextrin added to the filtrate, and the whole left at 37° in a thermostat for 7–8 hours. For experiments in aqueous solution, 10 cc. of 1 % amylase purchased from Merck and 0·5 g. of charcoal were taken. After filtration, 10 cc. of 1 % dextrin were added to the filtrates (Table IX). Over 90 % of the amylase present undergoes adsorption, and is present in suspension as an adsorption compound.

From Table X we see that the degree of adsorption of amylase is unaffected by the addition of such powerful elutive agents as butyl and propyl alcohols. Similar results were obtained using diastase from frog's blood. Here a slightly different experimental procedure was adopted. Instead of filtration, the solutions were centrifuged. Almost identical results were obtained with those mentioned above for Merck's amylase, *i.e.* strong adsorption, without elution by alcohols. The only difference is in the slightly lower degree of adsorption of frog amylase.

Table IX. *Mg. dextrin decomposed by filtered amylase solutions. Average figures for eight experiments.*

Without charcoal		With 0·5 g. of charcoal	
Average	Deviations	Average	Deviations
72·1	69·7–74·2	6·8	6·6–7·1

Table X. *Mg. of dextrin decomposed by filtered solutions of amylase. Average figures and deviations for six experiments.*

1 Without charcoal	2 0·5 g. charcoal	3 Charcoal and propyl alcohol	4 Charcoal and butyl alcohol	5 Propyl alcohol	6 Butyl alcohol	7 Charcoal filtered and propyl alcohol added to filtrate	8 Charcoal filtered and butyl alcohol added to filtrate
70·6	6·8	6·7	6·7	69·6	69·2	6·6	6·7
68·7–73·0	6·7–7·1	6·6–6·9	6·6–6·9	67·8–71·8	67·8–72·3	6·5–6·8	6·5–6·8

Differences in the velocity of hydrolysis of dextrin.

We have shown that the enzymic hydrolysis of dextrin proceeds in every case more rapidly in a homogeneous than in a heterogeneous system. The retardation of reaction increased with the amount of adsorbent added, until finally only 5 % of the hydrolysis taking place in a homogeneous system was observed.

Two factors might lead to this retardation: the enzyme might be inactivated by adsorption or the enzyme might be spatially separated from its substrate by the fixation of both on the adsorbent. Since we have evidence that adsorbed enzymes may still be active, it remained to establish which factor or factors enter into the present case. It appeared that in order to elucidate this point, certain properties of the enzyme in question would have to be considered. With this end in view, three types of experiments were undertaken. These had as their object to establish, firstly, that the addition of alcohols in such quantities as cause elution of substrate but not of ferment accelerates enzymic hydrolysis; secondly, that completely adsorbed amylase can catalyse the decomposition of dextrin; and thirdly, that the velocity of enzymic hydrolysis is proportional to the concentration of unadsorbed dextrin in solution, and is equal to that of the same concentration of dextrin in the presence of the same quantity of ferment in a homogeneous system.

In our experiments upon the influence of alcohol, the concentrations of substrate, ferment and charcoal were the same as before. Four determinations were made of hydrolysis in the systems charcoal-amylase-dextrin and amylase-dextrin, with and without the addition of butyl and propyl alcohols in such concentration as to cause no elution of the ferment.

Table XI. *Mg. of polysaccharide decomposed. Average figures.*

			1 % amylase-charcoal-polysaccharide				
Substrate	No. of experiments	Time hours	Without alcohol	Butyl alcohol	Propyl alcohol	Charcoal g.	Amylase-polysaccharide
Dextrin	3	6	32·1	47·8	46·1	0·2	55·5
	3	6	16·1	28 1	26·4	0·4	55·2
	3	12	63·9	79·0	76·6	0·2	102·7
	3	12	24·1	36·3	37·2	0·4	103·4
Glycogen	2	6	10·8	16·7	17·1	0·4	46·9
	3	6	26·7	31·2	33·0	0·2	44·7
	3	12	19·9	32·0	31·8	0·4	80·2

The results shown in Table XI indicate that the addition of alcohols considerably accelerates the velocity of hydrolysis of polysaccharides. This effect can be due only to an increase in the concentration of unadsorbed dextrin. It hence follows that, in the presence of substances which adsorb both enzyme and substrate, the addition of alcohols or other narcotics leads to an augmentation of the velocity of reaction due to elution of the substrate, but not of the ferment.

It remains to be proved, however, whether the adsorbed ferment has any hydrolytic action upon dextrin. Thus, in the above case, the possibility may exist that, both in the presence or absence of alcohols, only the unadsorbed portion of the enzyme has any action, differences in velocity of hydrolysis being due exclusively to elution of substrate. We see, however, no *a priori* reason for believing that adsorption should inactivate an enzyme, unless, of course, attachment to the adsorbent binds some group indispensable to the action of the ferment.

In order, however, to establish that amylase remains active after adsorption, the following experiments were conducted. 1 g. of charcoal was added to 10 cc. of 0·5 % amylase, and the mixture shaken mechanically at 25° for two hours, after which it was centrifuged and the supernatant liquid completely poured off. The residue was then washed five times with water at 25°, glycogen solution was added, and the whole left for from 2 to 4 hours at 37°. We know from Unna's experiments that under these conditions adsorbed amylase is not eluted by glycogen, so that any hydrolytic action observed must be due solely to the adsorbed ferment. Table XII shows that the hydrolytic action of such enzyme-adsorbent preparations upon glycogen is quite considerable.

Table XII. *Non-elution of amylase by polysaccharides.*

Substrate	No. of experiments	Mg. reducing sugars found after contact of from 2–8 hours of adsorbed ferment with substrate		Mg. reducing sugars produced in solution after centrifuging and decanting
		Average	Deviations	
Dextrin	6	21·8	10·6–31·5	0
Glycogen	4	26·3	11·5–33·2	0

That longer contact of adsorbed amylase with glycogen solution does not lead to elution of ferment is shown by the following type of experiment.

The concentration of reducing sugars found after a 4–8 hours' contact of glycogen solution with the charcoal-amylase preparation is determined, and the mixture centrifuged. A portion of the clear supernatant liquid exhibits no increment in the amount of reducing sugars present after a further lapse of 6–8 hours (cf. Table XII).

We may hence consider the activity of adsorbed amylase to be conclusively established, as also its non-elution by glycogen solutions.

The above result is, however, only a proof that adsorbed amylase possesses enzymic activity; whether this activity is as great as that of the free ferment remains to be proved.

Thus the retardation of hydrolytic action in systems of the type amylase-charcoal-polysaccharide may be ascribed either to depression of enzyme action due to adsorption, or to diminution in the concentration of free substrate owing to its fixation on the adsorbent. With a view to the elucidation of this question, the following experiments were carried out.

Charcoal and dextrin solution were added to a solution of amylase at 0°, and after 20 minutes the mixture was filtered, and the dextrin content of the filtrate determined. Two further portions were then prepared, one of which was identical with the above, whilst the other from which charcoal was absent contained an identical concentration of amylase but a concentration of dextrin equal to that found in the filtrate in the preliminary experiment. Both portions were placed in a thermostat and their reducing sugar content was determined after from 2 to 8 hours.

Concordant results were obtained from a number of experiments in which the velocity of hydrolysis was equal in both cases (cf. Table XIII). It hence follows that the efficiency of adsorbed amylase is quantitatively equal to that of the free enzyme.

Differences in the velocity of hydrolysis of polysaccharides by amylase, caused by the addition of charcoal to the system, can therefore be ascribed solely to the spatial separation of the enzyme from its substrate by this fixation on the adsorbent.

Table XIII.

	Amylase-charcoal-dextrin		Amylase-dextrin	
No. of experiments	Average % dextrin unadsorbed	Average glucose found mg.	Average % dextrin	Average glucose found mg.
4	0·71	31·2	0·71	32·0
4	0·53	18·8	0·53	18·9
4	0·29	6·2	0·29	6·1
4	0·12	3·7	0·12	3·6

As to the question of the applicability of the law of mass action, it follows that the velocity of enzyme reaction is, within certain limits, independent of the absolute concentration of the substrate, but is proportional to that of the unadsorbed substrate. For this reason the velocity of reaction diminishes proportionately to the amount of adsorbent added (Table XIII).

SUMMARY.

1. The effect of heterogeneity of the medium upon the course of enzymic reactions is considered in relation to reactions in the living organism and is studied experimentally upon models consisting of enzyme, adsorbent and substrate.

2. In the case of the system urease-charcoal-urea, urease is shown to be almost quantitatively adsorbed, whilst urea is adsorbed only to a very small extent. The addition of propyl or butyl alcohol produces no measurable elution of the ferment.

3. In such a system, in which the ferment, but not the substrate, is adsorbed, the velocity of enzymic reaction is practically the same as in the absence of an adsorbent, and is unaffected by the addition of narcotics.

4. In the case of the system amylase-charcoal-glycogen or -dextrin, the ferment is, like urease, practically quantitatively adsorbed, whilst the percentage adsorption of dextrin or glycogen amounts to from 50 to 75 %, depending upon the quantity of charcoal added.

5. The addition of propyl or butyl alcohol, or of other narcotics, leads to an elution of about 50 % of the substrate; at the same time no measurable elution of ferment takes place. The percentage elution of polysaccharide by alcohols is greater, the greater the percentage adsorption.

6. In this system, in which both enzyme and substrate undergo adsorption, the velocity of enzymic hydrolysis is considerably lower than in the absence of an adsorbent.

7. This diminution in reaction velocity is explained as being due to the spatial separation of enzyme and substrate by fixation on different parts of the adsorbent surface.

8. The addition of narcotics to this system accelerates the reaction by increasing the concentration of unadsorbed substrate, which alone is able to enter into reaction.

9. The law of mass action is followed in enzyme reactions in a heterogeneous medium, with the reservation that the effective concentration of the substrate be taken as that part of the latter which is in solution.

10. The non-degradation of glycogen by frog's liver during the winter months is explained on the basis of the above findings, amylase and glycogen being supposed to be fixed on different parts of the structural surface of the liver cell.

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CXXXVII. STUDIES IN PURINE METABOLISM.

III. BASAL METABOLISM AND PURINE CONTENT.

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Basal metabolism has been found experimentally to be more nearly a function of body surface than of weight; this relation, the law of surfaces, is, as has been shown by Rubner [1883], Richet [1891], Frank and Voit [1901], Slowtzoff [1903], Lautanié [1905] and Benedict and Talbot [1915], only approximately exact. Further, since at thermal neutrality basal metabolism still remains a function of body surface, it would appear that thermolysis is not the cause of this constancy, but that this must be ascribed to some other factor. Very numerous explanations of the law of surfaces have been advanced. These may be divided into four groups—anatomical, physico-chemical, nervous and chemical. The first of these groups, represented by von Hösslin [1888], Terroine [1924, 1], and others, connects basal metabolism with the volume of blood passing a given area in unit time. The second group [Pfaundler, 1916, 1921] considers the cellular surface to be the deciding factor in thermogenesis, although Rubner's [1913] findings tend to discredit this view. The third group [Richet, 1889; Lefèvre, 1911; de Almeyda and de Fialtro, 1925] consider basal metabolism to be under nervous control.

The fourth group deals with chemical differences, and is mainly occupied by attempts at determining the "active mass" of an animal. According to these views, some portion of the complex chemical mechanism of the animal system is responsible for the energy output of the latter, the remainder serving various ends, such as structural, connective, and reserve tissues. If, therefore, the weight of this active portion of the animal could be determined, this should stand in some simple proportion to its basal metabolism.

Attempts at determining this active mass have been so numerous that I shall here cite only the more important papers on the subject. According to Terroine [1924, 2] neither the lipoid content, nor that of lipoid phosphorus can in any way be connected with basal metabolism. As to the mineral constituents, he finds that neither their total amount, nor the percentage of iron, which might be supposed to catalyse oxidations, can be considered to be the active mass, and in the same paper he states that there is no real reason for assuming purine content to be the active constituent in question, and concludes that, since thermal neutrality is a condition almost never met with under natural conditions, the law of surfaces is nevertheless valid and rational and that it is thermolysis which determines thermogenesis. In certain other papers, however [Terroine and co-workers, 1923, 1924, 1926], the opinion is put forward that the active mass of the body may be expressed by its protein content. Since this is proportional to the body weight only in animals divested of reserves of all types, this type of metabolism, where the energy output is constant per unit weight of animal, is met with only in starvation. Hence, Terroine, Feuerbach and Brenckmann [1924] concluded that the average animal contains two types of material; one a vital, active portion, of constant composition, unalterable by the exigencies of life, and the other, consisting of reserves, whose relative proportion is variable and which normally mask the active mass of the animal. In 1923, however, the same authors found that, since the proportion of protein present in animals so different as mice and bulls differs very little, whilst the basal metabolism per unit mass differs very widely, the protein content cannot be considered to be representative of the active mass.

Le Breton [1926] is, however, of the opinion that body-proteins are divisible into two classes—active protoplasm and inactive protoplasm, or paraplasm. According to this hypothesis, the size of an animal is determined by the inherent metabolic intensity of its active protoplasm, whose heat production must be equal to loss of heat from body-surfaces. If, by the inclusion of paraplasm in the cytoplasm the animal can dilute its active mass, it can then grow larger until thermal equilibrium is attained.

Terroine and Roche [1925], however, conclude from measurements of the intensity of respiration of minced tissues of homeotherms of different sizes that the metabolic intensity of protoplasm is identical in the animals studied. These authors therefore concluded that the intensity of basal metabolism depends, not upon differences in protoplasm, but upon blood supply.

Terroine, in one of the above cited papers [1923] expressed the opinion that nuclear matter is the active mass in question. Indeed, a number of biologists have drawn attention to the importance of the nucleus in cellular metabolism. Thus, Palladine [1896] considered that the respiration of plant tissues is proportional to their nuclear content, Loeb [1913] and Osterhout [1917] found that the nucleus is the seat of the most intensive oxidative processes taking place in living tissues, and Robertson [1923] thought that oxidative activity should be proportional to the nuclear-plasmic ratio of a given tissue or cell. Warburg [1909] showed that nucleated blood corpuscles consume more oxygen than non-nucleated, and that, further [1912], if a cytolysed suspension of avian erythrocytes be centrifuged, respiration is confined to the lower layer, in which may be found the nuclei. On these grounds, therefore, one might expect the basal metabolism of an animal to be proportional to the nuclear content of the latter.

In our opinion, the magnitude of the nuclear-plasmic ratio would be an even better index of metabolic intensity than merely the nuclein content, since it deals with only the actively reacting portions of the cell, excluding differences due to variable water and reserve contents among the various individuals examined.

Since we have been able to find no reliable values for the purine content or nuclear-plasmic ratio of various animals, this research was undertaken with a view to the confirmation or otherwise of the above hypothesis, connecting basal metabolism with purine content or nuclear-plasmic ratio.

EXPERIMENTAL.

Purine-nitrogen, which is taken as representative of nuclear content, the total nitrogen, and the nuclear-plasmic ratio were determined by the methods described in a previous paper [Truszkowski, 1926]. The animals examined were rats, guinea-pigs, rabbits, dogs, horses and cattle. The first two species were analysed whole, after removal of food remains from the alimentary tract and mincing. In the case of rabbits, dogs, horses and cattle, skeletal muscle and liver alone were analysed, since for technical reasons the analysis of whole animals larger than guinea-pigs presented too great difficulties: for comparative purposes, analyses were also made of the skeletal muscle of rats. It is realised that results obtained for one portion of an animal need not necessarily apply to the animal as a whole, but in view of the preponderance of muscular and glandular tissue among the nitrogenous constituents of the homeotherms, there is some justification for considering that the values obtained for muscle and liver are to a large extent representative of those which would be obtained for the entire animal.

The values for whole rats are taken from our previous paper (Part I, p. 441), and the mean values are given in Table II. Those obtained for skeletal muscle taken from six rats are shown in Table I. The water content, and total and purine-nitrogen are higher than for the whole animal, but the nuclear-plasmic ratio differs by less than 3 %. In the same table may be found the results obtained for guinea-pigs. Here the solid substance varies from 28 to 29·8 %, total nitrogen from 3·421 to 3·529 %, and purine-nitrogen from 72·18 to 77·47 mg. %; the nuclear-plasmic ratio varies from 21·56 to 23·63, on the average $22{\cdot}60 \times 10^{-3}$.

The values for skeletal muscle and liver of rabbits are taken from some unpublished results, which Dr Dmochowski has communicated to me. As these will be published separately, and deal with a different problem, only the mean values will be given here (Table II), and the same applies to those obtained by Miss Rowinska and Mr Zdunkiewicz for cattle and by myself for dogs. In the latter case, the animals weighed from 5 to 10 kg. and were fed on a diet poor in protein (oatmeal, potatoes, etc.).

In the case of horses (Table I) fairly wide variations were found; for muscle, total nitrogen varies from 3·119 to 3·550%, calculated for dry tissue from 13·32 to 15·40 %; purine-nitrogen is from 77·0 to 83·7 mg. %, and the nuclear-plasmic ratio varies from 22·17 to 26·48 × 10^{-3}. The liver has a lower water content (71·1 %) due obviously to the presence of some non-nitrogenous substances, since the nitrogen content is lower than in muscle, being 3·204 and 11·15 % for fresh and dry weight respectively. The purine content is considerably higher than for muscle, and is, on the mean of two determinations, 147 mg. %, and the nuclear-plasmic ratio, which shows wide variations for liver tissue in general, is in one case 42·12 and in the other 54·89, on the average 48·50 × 10^{-3}.

Table I.

Species	No.	Material taken	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live weight	Purine-N mg. % dry weight	Nuclear-plasmic ratio
Rat	1	Skeletal muscle	28·0	3·850	13·90	81·7	294·9	21·68 × 10^{-3}
,,	2	,, ,,	28·0	3·842	13·80	78·2	282·2	20·76 × 10^{-3}
Guinea-pig	1 ♂	Whole	29·8	3·447	11·67	80·3	269·5	23·63 × 10^{-3}
,,	2 ♂	,,	28·0	3·529	12·57	77·5	276·7	22·51 × 10^{-3}
,,	3 ♀	,,	28·0	3·421	12·22	72·2	257·7	21·56 × 10^{-3}
Horse	1 ♀	Skeletal muscle	20·8	3 188	15·32	—	—	—
,,	2 ♂	,, ,,	24·9	3·550	14·26	77·0	309·2	22·17 × 10^{-3}
,,	3 ♂	,, ,,	23·4	3·119	13·32	—	—	—
,,	4 ♂	,, ,,	23·2	3·142	13·54	81·0	349·3	26·48 × 10^{-3}
,,	5 ♀	,, ,,	21·8	3·357	15·40	83·7	383·8	25·57 × 10^{-3}
,,	6 ♀	,, ,,	21·8	3·342	15·30	81·9	375·8	25·14 × 10^{-3}
,,	3 ♀	Liver	30·3	2·987	9·86	155·4	513·1	54·89 × 10^{-3}
,,	4 ♂	,,	27·5	3·421	12·44	138·3	502·9	42·12 × 10^{-3}

The mean values obtained by other authors and by myself are given in Table II. Rats and guinea-pigs give fairly concordant values, all figures obtained for the latter being slightly higher than for the former, with the exception of the percentage of solid substance. Thus, for whole animals, it would not appear that either purine content or nuclear-plasmic ratio is in any way connected with basal metabolism.

For muscular tissue much more divergent results are found.

Table II. *Mean values.*

Species	Material taken	% solid substance	% N live weight	% N dry weight	Purine-N. mg. % live weight	Purine-N. mg. % dry weight	Nuclear-plasmic ratio	Author
Rat	Whole	33·45	3·285	9·745	65·2	196·0	20·62 × 10^{-3}	Truszkowski
Guinea-pig	,,	28·6	3·466	12·152	76·7	268·0	22·60 × 10^{-3}	Truszkowski
Rat	Skeletal muscle	27·7	3·846	13·85	80·0	288·6	21·22 × 10^{-3}	Truszkowski
Rabbit	,, ,,	24·5	3·777	15·41	115·5	471·3	31·54 × 10^{-3}	Dmochowski
Dog	,, ,,	21·2	2·514	12·06	54·2	264·0	22·01 × 10^{-3}	Truszkowski
Horse	,, ,,	22·6	3·283	14·53	80·9	358·8	25·33 × 10^{-3}	Truszkowski
Cattle	,, ,,	25·3	3·933	15·58	76·5	284·1	19·86 × 10^{-3}	Rowinska and Zdunkiewicz
Rabbit	Liver	26·3	2·819	9·85	140·0	536·3	52·25 × 10^{-3}	Dmochowski
Dog	,,	24·4	2·335	9·65	86·7	355·5	40·53 × 10^{-3}	Truszkowski
Horse	,,	28·9	3·204	11·15	146·9	508·0	48·50 × 10^{-3}	Truszkowski
Cattle	,,	24·8	3·115	12·56	118·8	478·9	39·71 × 10^{-3}	Zdunkiewicz

No proportionality between size of animal and water, nitrogen, or purine content of muscles, or of their nuclear-plasmic ratio, appears to exist. Thus, whilst rat muscle has the highest percentage of solid substance, that of cattle is the next largest, then that of rabbits, horses and dogs. The nitrogen content decreases in the order—cattle, rats, rabbits, horses, and dogs; and the purine content in the order—rabbits, horses, rats, cattle and dogs. The nuclear-plasmic ratio is highest for rabbits, *i.e.* $31{\cdot}54 \times 10^{-3}$, then horses, dogs, rats and cattle, $19{\cdot}86 \times 10^{-3}$. Thus we see that rats and cattle, whose average weights are as 1 : 2500 are the most similar to each other in purine and nitrogen content of muscle, and that generally, no regularity exists in the values obtained for the above series of animals.

The same may be observed in the values found for the liver. Here horses have the highest percentage of solid substance, next rabbits, cattle and dogs. The highest nitrogen content may be found in horses, then cattle, rabbits and dogs, whilst the purine nitrogen content decreases in the order—horses, rabbits, cattle and dogs. The nuclear-plasmic ratio is, as for muscle, highest in the rabbit ($52{\cdot}25 \times 10^{-3}$), then follow horses, dogs and cattle ($39{\cdot}71 \times 10^{-3}$). Here, also, there is no connection between the values obtained and the weight, *i.e.* the basal metabolism of the animal.

It is, however, interesting that the liver has a nuclear-plasmic ratio approximately double that of skeletal muscle, and this is in accordance with histological findings for the same tissues. No particular stress is laid upon the values other than this ratio, since it is realised that these depend upon the state of nutrition of the given animal at the moment when killed, and to a certain extent upon the previous history of the latter, which is, in the case of horses and cattle, unknown.

DISCUSSION.

The results given above show that considerable variations may exist in the nitrogen and purine contents of the liver and skeletal muscle of various animals and in their nuclear-plasmic ratios, but that these variations are in no way connected with the basal metabolism of the given animal. The values obtained are, however, to a certain extent constant for a given species, and appear, further, to be characteristic of the particular organ examined.

There exists, however, the possibility that, whilst basal metabolism does not depend upon nuclear mass, it may depend upon the active surface of the nucleus. Thus, admitting that reactions of oxidation take place within the nucleus, their intensity will depend not so much upon its mass as upon the nuclear-plasmic intersurface. It is therefore conceivable that equal nuclear masses need not be equivalent, their activity depending upon the development of their surfaces. This, again, may be influenced by two factors, namely, cell dimensions and concentration of active substances within the nucleus.

In the first case, it is obvious that the smaller the cell, the greater the relative nuclear surface, assuming the volumes of nucleus and plasma to diminish proportionally. Unfortunately very little work has been done in this field, and those values which I have been able to find are contradictory. Amelung [1893] and Strasburger [1893] found that cells of the same organ in plants of different size but of the same species are of equal size. Rabl [1899] found that the dimensions of epithelial cells and lens fibres were the same for dogs of various sizes. On the other hand, Levi [1905], found that sections of cerebral cells of different mammals were greater, the greater the weight of the animal, that of the ox being 104·3μ, whilst that of the mouse is 37·25μ, and differences of the same order may be found for lens fibres. These differences are, however, insufficient to explain differences in basal metabolism, since, if we assume that the nuclei diminish in radius to the same degree as the cells, this would mean that the surface of unit weight is approximately three times greater for mouse than for ox nuclei, whereas the basal metabolism of the former animal is about 20 times as great as for the latter. However, in view of the lack of reliable data on this point, it is unprofitable further to discuss it, and the same applies to the possibility of enlargement of nuclear surface by dilution.

SUMMARY.

1. The chemical nuclear-plasmic ratios of whole rats and guinea-pigs differ little from one another.

2. The values obtained for purine- and total nitrogen, and for the nuclear-plasmic ratios of liver and of skeletal muscle of rats, rabbits, dogs, horses and cattle vary within wide limits, but are in no way connected with the basal metabolism of the animals in question.

3. The purine content of animals cannot therefore be identified with their "active mass."

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CXXXVIII. STUDIES IN PURINE METABOLISM.

IV. THE NUCLEAR-PLASMIC RATIO IN DOGS IN CARBOHYDRATE AND PROTEIN FEEDING, AND IN STARVATION.

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In Part I of this series [1926] it was shown that the nuclear-plasmic ratio of rats is slightly lowered on death from total inanition, whence it was concluded that protein reserves do not exist in the cytoplasm of rats. In this paper similar measurements made on the liver and skeletal muscles of dogs under various conditions of alimentation will be described.

Whilst the theoretical and historical aspects of the question of protein reserve were considered in the above-mentioned paper (Part I), as certain other researches have since come to my notice, I shall commence with their discussion.

Terroine and co-workers have, in a series of papers on inanition, expressed their views on the protein reserve question. Thus Terroine, Feuerbach and Brenckmann [1923] considered that, as the percentage protein content of mice and small birds increases during inanition, protein reserves cannot exist in these animals. In 1926, Terroine, Trautmann and Schneider express the opinion that, if protein reserves do exist, they must be very small in comparison with carbohydrate and fat reserves. Since, however, they found an increase in the total nitrogen content of chicken liver and muscle after protein feeding, they conclude that a limited storage of proteins may exist.

A number of other authors such as Friske [1909] and Junkersdorf [1921] have observed that the nitrogen content of various organs rises in protein feeding as compared to carbohydrate feeding, and have hence drawn the conclusion that this is due to protein storage.

The experiments conducted in this paper show, however, that the premises upon which such arguments are based are misleading, since no analysis is made of the repartition of this nitrogen between nucleus and cytoplasm. This question will be discussed more fully later.

Rubner [1911] describes an experiment of Thomas in which, in a man maintained on a carbohydrate diet after a period of protein feeding, nitrogen elimination falls for nine days, and this Lusk [1924] explains as being due to the gradual solution of "deposit protein," which may amount to 3 % of total proteins. A very detailed study of nitrogen hunger in man has been published by Smith [1926], whence it appears that, whilst uric acid-nitrogen excretion is practically constant throughout the period of nitrogen hunger, total nitrogen falls constantly during the experimental period of over three weeks; whilst at first sight this research would appear to support the view that protein storage exists, we shall adduce evidence in a later part of this paper that the results of this author might be interpreted otherwise.

Finally, Tichmeneff [1914] and Cahn-Bronner [1914; Berg and Cahn-Bronner, 1914], on the basis of metabolism experiments, conclude that the liver may serve as a storehouse of protein matter.

It is our opinion that metabolism experiments alone, unsupplemented by direct analyses, cannot yield decisive results in such cases. In particular, the question of the fate of retained nitrogen cannot be answered merely by drawing up a nitrogen balance sheet, whilst the evidence on which protein storage in mammals is accepted is extremely slender.

In order to throw further light on the above question, analyses have now been made with the object of determining the total nitrogen, protein and purine-nitrogen contents and the nuclear-plasmic ratio of the liver and skeletal muscles of dogs on a carbohydrate and a protein diet, and in inanition. Should any protein be stored in the cytoplasm of the cells of the above tissues, one would expect the ratio of nuclear to cytoplasmic nitrogen to fall, since we have no reason to believe that reserve protein is a nucleoprotein. Similarly, if in hunger reserve protein is used up, the nuclear-plasmic ratio should rise accordingly.

EXPERIMENTAL.

A series of dogs was fed for three weeks on oatmeal and potatoes—a diet which contains sufficient protein to cover nitrogen catabolism, but which is mainly carbohydrate. The dogs were then killed by injection of chloroform into the heart, the liver and about 100 g. of mixed skeletal muscle being then removed. The liver, after expression of blood and washing, was weighed and minced; from the well mixed mince, 50 g. were taken for hydrolysis and 20 g. were dried to constant weight. In certain cases a further 20 g. portion was extracted with hot alcohol and dried. Portions of the powdered residue were then weighed out, and nitrogen determined by the Kjeldahl method. In this way the percentage of protein nitrogen could be determined, as distinct from total nitrogen, which was determined from the hydrolysate of the 50 g., as described in a previous paper [Truszkowski, 1926]. Purine-nitrogen was determined by the method of Krüger and Schittenhelm [1905] and the nuclear-plasmic ratio was calculated as $N_P/N_T - N_P$.

Skeletal muscle was taken from all four limbs and from various parts of the body; as far as possible fat and sinews were removed, and the remainder minced and treated as for liver, except that protein-nitrogen was not separately determined.

The results obtained are shown in the first parts of Tables I and II. Table I gives the values found for liver. The weight of this organ varies from 151 to 320 g., this depending largely on the weight of the dog examined. Percentage dry substance varies from 20·7 to 25·9, average 24·4. The lowest nitrogen content is 1·898 %, and the highest 2·800 %; for dry weight from 7·33 to 11·46 %. Protein-nitrogen, as determined for dogs 1, 4 and 5 is almost identical with total nitrogen in the first case, and 10 and 5 % higher in the other two cases respectively, indicating an extraction of non-nitrogenous constituents, probably lipins, by the alcohol. Purine-nitrogen varies from 76·4 to 93·3, average 86·7 mg. % live weight and from 325·5 to 396·8, average 355·5 mg. % dry weight. The nuclear-plasmic ratio varies from 32·71 to 47·26—a maximum deviation of about 20 % above and below the average of 40·53 × 10^{-3}.

Table I. *Liver in carbohydrate and protein feeding, and in starvation.*

No.	Weight of liver g	% of dry substance	% N live weight	% N dry weight	% Protein-N live weight	% Protein N dry weight	Purine-N mg. % live weight	Purine-N mg % dry weight	Nuclear-plasmic ratio × 10^{-3}	Nature of feeding
1 ♂	240	25·6	2·055	8·03	1·979	8·097	89·9	350·9	45 69	3 weeks carbohydrate
2 ♀	267	25·9	1·898	7·33	—	—	84·3	325·5	46·46	3 ,, ,,
3 ♂	329	25·4	2·837	11·16	—	-	89·9	353·6	32·71	3 ,, ,,
4 ♂	151	25 2	2·800	11·14	—	12·30	—	—	—	3 ,, ,,
5 ♂	231	20 7	2·372	11·46	—	11·93	76 4	369·2	33·30	3 ,, ,,
6 ♂	320	23·5	2·066	8·79	—	-	93·3	396·8	47·26	3 ,, ,,
Mean	—	24·4	2·335	9 65	—	-	86·7	355·5	40·53	
7 ♀	225	24·3	3·311	13·62	—	—	124 4	511·8	39 02	3 weeks meat
8 ♂	280	29·5	3·334	11·31		-	—	—	—	3 ,, ,,
9 ♂	325	22·7	2·847	12 54	—	—	128·3	565·2	47·19	10 ,, ,,
10 ♂	383	26·5	4·489	16·94	3·303	13·62	144·7	546·2	33 33	12 ,, ,, *
11 ♀	840	24·0	2·926	12·19	—	—	114 5	476·9	40·70	12 ,, ,,
Mean	—	25·4	3·341	13·32	—	—	127·5	525·0	39·69	
12 ♂	141	25·2	4·064	16 11	3 929	16·20	167·6	663·7	43·02	14 days manition
13 ♂	200	24·3	3 181	13·09	—	—	123·8	509·3	40·49	23 ,, ,,
14 ♂	86	26·5	3·931	14 84	—	—	182·9	690 2	48·79	35 ,, ,,
Mean	—	25 35	3·725	14 68	—		158·1	621·1	44 10	

* Dog No. 10 was killed by strychnine.

A second series of dogs was fed for periods of from 3 to 12 weeks on boiled horseflesh, and the same values determined as before (Tables I and II. second portions). Here we may note that the weights found for the liver are on the whole greater, although the dogs were about the same size. The percentage dry substance is also slightly greater, total nitrogen is 43 and 38 % higher, and purine nitrogen 47 and 41 % higher for wet and dry weights respectively. As a result the nuclear-plasmic ratio shows a slight increase of from 38·58 to 39·69 × 10^{-3}. One dog of this series was killed by strychnine (No. 10), and this dog shows exceptionally high values for total nitrogen, although protein-nitrogen gives figures comparable with the total nitrogen found in the other four cases. These high values may be partly due to loss of glycogen from the liver, and partly to the presence of extractive non-protein-nitrogen.

Three dogs were subjected to inanition, water being allowed freely. Two were taken for analysis immediately upon death from hunger, and one was killed whilst in the last stages of exhaustion. The duration of life under these conditions depended upon the room temperature, which was, however, never above 10°, and upon the initial state of nourishment. Here the percentage dry substance in the liver (Table I, Part III) is almost identical with that in meat feeding, total nitrogen is 10 % higher and purine-nitrogen about 20 % higher. Protein-nitrogen, as determined for dog No. 12, is almost identical with total nitrogen. The nuclear-plasmic ratio is about 10 % above that found in carbohydrate and protein feeding, pointing to a greater depletion of non-purine than of purine constituents of the liver.

Table II shows, on the whole, a similar state of affairs in muscle to that in liver, under the same conditions.

Table II. *Skeletal muscle in carbohydrate and protein feeding, and in starvation.*

No.	% solid substance	% N live weight	% N dry weight	Purine-N mg. % live weight	Purine-N mg. % dry weight	Nuclear-plasmic ratio $\times 10^{-3}$	Nature of feeding
1 ♂	21·1	2·398	11·36	47·1	223·2	20·03	3 weeks carbohydrate
2 ♀	24·5	2·575	10·51	58·0	236·9	23·07	3 ,, ,,
3 ♂	21·3	2·742	12·88	58·2	273·6	21·70	3 ,, ,,
4 ♂	21·8	2·531	11·61	55·1	252·8	22·26	3 ,, ,,
5 ♂	18·0	2·310	12·83	49·2	273·3	21·76	3 ,, ,,
6 ♂	20·3	2·527	12·47	57·4	283·4	23·25	3 ,, ,,
Mean	21·2	2·514	11·88	54·2	256·2	22·01	
7 ♀	26·3	3·181	12·09	72·1	274·0	23·18	3 weeks meat
8 ♂	22·3	3·087	13·85	84·3	377·9	28·06	3 ,, ,,
9 ♂	24·8	3·357	13·54	87·7	353·8	26·84	10 ,, ,,
10 ♂	23·8	3·931	16·52	81·7	343·4	21·23	12 ,, ,,
11 ♀	24·0	3·480	14·50	77·3	322·1	22·64	12 ,, ,,
Mean	24·2	3·407	14·10	80·6	334·2	24·28	
12 ♂	18·8	3·174	16·88	—	—	—	14 days hunger
13 ♂	24·3	3·527	14·52	83·68	344·3	24·30	23 ,, ,,
14 ♂	19·5	3·249	16·66	65·66	336·7	20·63	35 ,, ,,
Mean	20·8	3·317	16·02	74·67	340·5	23·02	

In meat feeding, dry substance is 14 % higher than in carbohydrate feeding, total nitrogen is 36 and 19 %, and purine-nitrogen 49 and 30·4 % higher for wet and dry weights respectively. Here, too, the nuclear-plasmic ratio is higher by 10 %, greater variations existing between the various values obtained in the meat than in the carbohydrate series, namely, from 20 to 23 in the latter and from 21 to 28 in the former. This difference is due to variable total nitrogen contents in the meat series, from 3·087 to 3·931 %. In hunger, the percentage solid substance falls to slightly below the carbohydrate level, with a corresponding drop in total nitrogen content, as a result of which the latter value for dry muscle is the highest of all obtained, corresponding to practically pure protein. The purine content is slightly lower than in meat feeding, and the nuclear-plasmic ratio lies between the two previously obtained values, showing that in dog muscle the ratio between cytoplasmic and nuclear nitrogen is constant, in spite of considerable variations in the individual values obtained for total and purine-nitrogen.

DISCUSSION.

The above results show that in dogs the nuclear-plasmic ratio of liver and skeletal muscle is in both cases increased by liberal meat feeding as compared to carbohydrate feeding. Hence it follows that, in these circumstances, not only is no specific protein laid apart in the cytoplasm of the cells of the organs in question, but proportionally more nuclear substance is formed. A further significant difference which may be observed is the considerable rise in the nitrogen content of the muscle and liver in meat feeding, amounting to from 30 to 50 % for both nucleus and cytoplasm. This indicates that nitrogen retention may be due not only to hyperplasia but also to an actual higher concentration of nitrogen in the cells; this difference is, further, too great to be explicable on the basis of different glycogen content. That this is due partly to a diminution in water content is shown by the greater percentage content of dry substance, particularly in muscle. This dehydrating effect of a protein diet has been shown by Bischoff and Voit [1860], and the above results afford, therefore, a confirmation of this early work.

In starvation the water content of skeletal muscle is of the same order as in carbohydrate feeding, whilst the nitrogen and purine contents are only slightly below the meat level. The values found for the nuclear-plasmic ratio are not higher than those found in the previous series, so that here there is no evidence for the existence of protein reserves in muscle cells.

In the case of the liver, dilution cannot be observed in hunger, the mean value found for percentage of dry substance being higher than in carbohydrate feeding. All other values are higher than in the previous series; in particular, high values are found for purine-nitrogen, which is 19 % above meat value, whereas total nitrogen is only 11 % above. In this case, therefore, it would seem that the nucleus has lost proportionally less than the cytoplasm, and consequently that the liver cells may contain a limited store of reserve protein, whose amount cannot be increased by liberal protein feeding, and which is called upon in extreme emergency. On the whole, however, it appears that protein is stored by the body not as a specific deposit protein, but rather by the proliferation of cells, accompanied by greater protoplasmic content of the latter, this difference being over 40 % in the case of the liver, and close on 40 % in the case of skeletal muscle.

Viewed in this light, various arguments advanced in favour of protein storage to a large extent lose their point. Thus the experiments cited in the introduction (Junkersdorf, Friske, Terroine), in which the nitrogen content of muscle and liver rises after protein feeding, are confirmed, but as the increase affects both nucleus and cytoplasm to the same extent, they cannot be considered to afford any evidence whatsoever that a specific protein is laid down.

Nor can the gradual diminution in nitrogen excretion observed during specific nitrogen hunger be put forward as proof of protein storage. It has been shown in this paper that in protein feeding the cells may contain 40 % more nitrogen than in carbohydrate feeding. This 40 % probably corresponds to Rubner's [1911] "improvement quota," and appears to be used up in nitrogen hunger; at the same time nuclear material is, however, catabolised. In nitrogen equilibrium the nitrogen of the entire cell is excreted as urea and uric acid. In nitrogen hunger, on the other hand, amino-acids derived from the autolysis of body tissues are not deaminated but are used for the production of new protein in surviving cells; this re-utilisation of protein material appears to become more and more complete as the body cells approach the carbohydrate diet level of protein content. At the same time, uric acid excretion, which represents nuclear catabolism, should remain constant, since (cf. Part II) purines are synthesised with comparative facility by the mammalian organism, but are not available for resynthesis of nucleic acids if introduced into the blood-stream. The gradual fall in nitrogen elimination during nitrogen hunger, and the constant excretion of uric acid may, therefore, be explained without the necessity of introducing the conception of specific protein storage.

SUMMARY.

1. The chemical nuclear-plasmic ratio of the liver and of skeletal muscle of dogs has been determined, under various conditions of alimentation.

2. The total nitrogen and purine contents of the liver are respectively 43 and 47 % higher in dogs fed with meat than in those fed on carbohydrates.

3. The nuclear-plasmic ratio of liver tissue in meat-fed dogs is almost the same as on a carbohydrate diet, indicating that a liberal protein diet does not lead to the formation of specific deposit protein.

4. The content of solid substance is 14 % higher in skeletal muscle of dogs fed on a meat than on a carbohydrate diet, pointing to a certain dilution of the tissues in the latter case.

5. The total nitrogen and purine contents of skeletal muscle are respectively 35 and 49 % higher in dogs fed with meat than in those fed on carbohydrates.

6. The nuclear-plasmic ratio of skeletal muscle in meat-fed dogs is slightly higher than that encountered in carbohydrate feeding; hence specific reserve protein is not laid down in the muscles as a result of exclusive protein feeding.

7. In starvation, the nuclear-plasmic ratio of the liver is 10 % above normal, pointing to the possible presence of specific reserve protein in this organ.

8. Under similar conditions, the nuclear-plasmic ratio of skeletal muscle is unchanged, indicating that deposit protein is absent from this tissue.

In conclusion, I wish to acknowledge my gratitude to Prof. St. J. Przylecki, who suggested this research and whose constant help and advice were indispensable to its accomplishment.

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CXXXIX. REACTION OF FATTY EXTRACTS OF CERTAIN ORGANS WITH THE ANTIMONY TRICHLORIDE TEST FOR "VITAMIN A."

(PRELIMINARY NOTE.)

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THE test as described by Carr and Price [1926] is thought to indicate the presence of the fat-soluble vitamin A. It is carried out as follows: 0·2 cc. of a 20 % solution in chloroform of the fat to be tested is added to 2 cc. of a 30 % solution of $SbCl_3$ in chloroform. A blue colour results which varies in depth with the amount of the reacting substance present; the colour fades rapidly, the time taken for complete disappearance depending on the depth of the original colour.

It appeared to be of interest to examine the comparative amount of the reacting substance contained in the fatty mixture extracted from the organs, in particular the liver, of man and other mammals without necessarily assuming this to be vitamin A.

Method.

(1) From 100 to 200 g. of liver, or other organ, was passed through a mincing machine into three times the volume of 94 % alcohol, the purpose of the procedure being mainly to facilitate the subsequent drying. The alcoholic extract (holding about 77 % alcohol) was found to contain traces of the reacting substance; the amount lost was however negligible.

(2) After 24 hours, during which time the material was occasionally stirred, the residue was strained off, squeezed out in a cloth and dried in a hot-air oven at about 95°.

(3) The dried material was ground to a fine powder in a mill, the powder being then extracted with ether (at room temperature in a flask) for 24 hours. At the end of this time the residue was filtered off and washed with ether on the filter.

(4) The filtrate was collected in a weighed basin, evaporated until there was no further smell of ether and weighed. It may here be mentioned that the yield thus obtained from different specimens of liver was from 1·3 to 2·7 % of the weight of organ dealt with. The method of extraction by infusion in cold ether with occasional shaking appeared to be sufficiently complete for

the purpose aimed at, which was to compare the amounts of reacting substance present in different fatty extracts.

(5) The fatty extract was dissolved in chloroform to make a 2, 10 or 20 % solution, the concentration being determined by the result of a preliminary test, carried out as described below, to show the comparative richness of the extract in the reacting substance.

The extracts were of a brownish yellow colour of varying depth; those from human liver and other tissues had the consistence of a thick oil but those from ox and sheep's liver were solid at 25°.

The intensity of the reaction with $SbCl_3$ had no relation to the depth of coloration of the extracts.

The liver extracts were tested for the presence of bile pigments with alcoholic iodine, the result being negative. The absence of any change of colour with iodine indicated also that carotene was not present.

In testing the fatty extracts of liver for bile pigment a glass rod wetted with fuming HNO_3 was drawn across a thin layer of the extract on a white porcelain surface; a very deep purple to a bluish pink colour was obtained, fading rapidly to pink and then disappearing. The depth of colour had no relation to the darkness of the brownish yellow coloration of the extracts but was found to be roughly proportional to the intensity of the reaction with $SbCl_3$. The reaction (with HNO_3) was therefore used as a preliminary test to determine the probable richness of different extracts in vitamin A.

To obtain a quantitative expression of the comparative amount of the reacting substance in extracts from different specimens, the blue colour produced with $SbCl_3$ was matched against solutions of indigo-carmine of known concentration. The standard taken was a sample of cod-liver oil which, when tested in 20 % solution, gave a colour matched by a $\frac{1}{5000}$ solution of indigo-carmine. This was given unit value, the content in vitamin A being expressed in multiples or fractions of the unit.

A colorimeter was not used, the comparison being made in test-tubes of the same internal diameter.

It appeared to be of interest to examine the unsaponifiable residue of the liver extracts. Two experiments were done, saponification being effected by boiling with alcoholic potash under a reflux condenser. In the first the saponified material was extracted with ether, the ether evaporated and the residue dissolved in $CHCl_3$; it gave a very strong reaction with both HNO_3 and with $SbCl_3$. In the second 15 g. of the mixed fatty extracts from different specimens of liver were saponified and the material was extracted with light petroleum. This was evaporated, an almost colourless residue being obtained of which 0·02 g. was soluble in $CHCl_3$. Tested in 0·2 % solution this residue had a unit value of 100. It contained cholesterol, the amount of which was not determined. The material thus obtained, although evidently very rich in the reacting substance, gave only a faint reaction with fuming nitric acid. To find some explanation for this rather unexpected result, the saponified liquid, which had already been extracted with light petroleum, was further extracted with ether, a yellow residue being obtained on evaporation of the ether which gave a strong colour reaction with HNO_3 and a comparatively slight reaction with $SbCl_3$.

In spite therefore of the apparent correspondence of the results of the two tests, when applied to the fatty extracts examined, the reactions appear to be due to the presence of two different substances. Both are found in the unsaponifiable residue, the substance reacting with $SbCl_3$ being however very much more soluble in light petroleum than is the substance giving a purple colour with HNO_3.

The method of colorimetry used would not serve for exact measurements; it gives however a comparative numerical expression of the content in vitamin A of the extracts which is approximately correct.

The subjoined table gives the results of observations on the extracts of human or other origin and of some other materials.

Table I.

Various extracts.

Source of extract	Value in vitamin A	Source of extract	Value in vitamin A
Cod-liver oil A	1	Kidney. Human	Trace
„ B	0·5	Liver. Guinea-pig	3·5
„ C	0·25	„ Ox (*a*)	10
Butter. Foreign	0·05	„ Ox (*b*)	11
„ Local (Cairo) 2 samples	0	„ Water-buffalo	6
Human Milk (Fats) 8	0·05–0·025	„ Camel	12·5
Heart. Sheep	0	„ Sheep (*a*)	7·5
Suprarenal. Ox	0·33	„ Sheep (*b*)	8
Spleen. Human	0	Snail (from 20 glands)	0
Placenta. Human, 2	0		

Extracts of Human Liver.

Source and cause of death	Value in vitamin A	Source and cause of death	Value in vitamin A
1 Child. Bronchopneumonia ...	25	13 Man. Pneumonia	2·5
2 Man. Phthisis	25	14 „ Pneumonia	2
3 Child. Burns	16	15 Child. Bronchitis	2
4 Man. Electric shock ...	12·5	16 Woman. Burns	1·5
5 „ Stenosis of larynx ...	12·5	17 Man. Pneumonia	1
6 „ Heart failure	10	18 „ Nephritis	0·83
7 „ Pneumonia	6	19 „ Aortic disease (syphilitic)	0·4
8 Woman. Burns	5	20 Man. Asphyxia	0·25
9 Child age 3 days. Exposure.	5	21 Child. Ill-developed, age 2 months. Corneal ulcers ...	0·2
Meconium from same ...	0	22 Man. Pellagra	0·1
10 Child (premature) 8 days. Pneumonia	3·5	Unsaponifiable residue of mixed liver extracts ...	100
11 Child. Acute enteritis ...	4·5		
12 Man. Heart failure	3·5		

Note. In regard to some of the results recorded in the table it is to be noted that a negative result with Carr's test does not demonstrate the absence of vitamin A, traces which are shown by the $AsCl_3$ test of Rosenheim and Drummond [1925] not being detected with $SbCl_3$.

My colleague Dr A. Hassan has been good enough to carry out some comparative experiments with the two tests which indicate that the former ($AsCl_3$) is between two and three times as sensitive as the latter but that in regard to the determination of the relative richness in vitamin A of different specimens of fatty extracts the two tests agree in their results.

It would be entirely premature to draw any conclusions as to the importance or otherwise of the amount of vitamin A found in the liver in relation to the diseases tabulated. In most of the cases no information was available as to the nature of the diet. The majority of the individuals had either been in hospital for too short a time for the diet supplied to have had any influence or had died outside hospital under conditions which necessitated a medico-legal *post mortem* examination. It may however be of interest to consider very briefly the cases in which there appears to be a great deficiency of vitamin A in the liver extract. These are numbered 20, 21 and 22.

Case 20. This case was that of a man who was buried by a fall of sand when excavating in a sand-pit. There was no evidence of what disease if any he may have suffered from. He belonged to the poorer class and it is unlikely that his diet differed much from that of many of the other cases recorded in whom the liver was found to be rich in vitamin A.

Case 21. The condition of the cornea in this case was suggestive of a deficiency in vitamin A. The child had scarcely grown, although for a month previous to death it had been under treatment in the Children's Institute at Kasr el Aini Hospital. While in hospital the child received partly artificial, partly breast-feeding (a healthy wet-nurse) with only a temporary increase in weight.

Case 22. This was a case of advanced pellagrous insanity, the man having been an inmate of the Khanka Asylum for a long period before his death. I have to thank Dr Miller, the Director of that institution, for kindly supplying the material. The amount of fatty material extracted was 2 % of the weight of liver taken, about the normal quantity.

The great deficiency of vitamin A in the liver extract need not be assumed to have any etiological connection with the disease (pellagra); to establish this the examination of a sufficient number of early cases would be required. It is in fact more probable that this deficiency is related to the fact demonstrated by Roaf and the writer [1918] in an experiment on five comparatively early cases of pellagra that the absorption of fats from an ordinary mixed diet is deficient to the extent of about 19·5 % of the fat intake. In an advanced case, such as No. 22, the extreme atrophy of the mucosa of the small intestine, in particular of the villi, would doubtless exaggerate the defective absorption.

If the substance giving the reaction be regarded as identical with vitamin A[1], the observations on the livers of animals used for food would indicate the value of liver as a foodstuff, especially for children. In the cases recorded in the table (ox, sheep, camel, water-buffalo) the average percentage of fatty extract was 2·5 %, the average value of which in vitamin A was 9·1 %. On the basis of these figures 100 g. of liver would have as regards vitamin A the same value as 22·5 g. of cod-liver oil.

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[1] Apart from the evidence given by Carr and Price [1926] for the identity of the reacting substance with the vitamin, the following facts are in support of the view: (*a*) the results with the $AsCl_3$ and $SbCl_3$ tests give comparable estimates of the amount of the reacting substance present; (*b*) the substance giving the reaction is gradually destroyed (in solution) by exposure to diffused daylight and air at the laboratory temperature; (*c*) the substance is found in the unsaponifiable residue. Wokes and Willimott [1927] in a recent paper on the effect of heat and oxidation on cod-liver oil state that in their observations with different colour tests for vitamin A the $AsCl_3$ and $SbCl_3$ tests were in agreement when used quantitatively.

SUMMARY.

Although in many respects the observations must be regarded as of a preliminary nature, the following conclusions appear to be justified.

1. The fatty extract from the human liver contains a substance giving the same colour reactions as vitamin A in an amount which may be equal to 25 times that found in a good sample of cod-liver oil.

2. The amount of vitamin A found in the liver varies within wide limits and may, under some conditions of disease, be no more than $\frac{1}{250}$ of the maximum amount observed to occur.

3. The substance is present in considerable amount in the liver at birth but is not present, in an amount detectable by the test used, in the placenta.

4. The extract of livers of healthy animals slaughtered for food was found to contain from 6 to 12·5 times the amount of vitamin A present in cod-liver oil.

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CXL. ON THE PIGMENT PRODUCED BY *CHROMOBACTERIUM VIOLACEUM.*

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(*Received July 31st, 1927.*)

THE bacterial pigments in general have received very little attention and any systematic investigations in this field have been confined mainly to the pigments produced by pathogenic organisms such as *Bacillus pyocyaneus.* The small amount of pigment formed in most cases is no doubt responsible for this. The extraordinarily intense development of pigment produced in a sample of milk through the growth of *Chromobacterium violaceum*, to which our attention was directed, suggested that this was a pigment which could be produced in sufficiently large quantities for examination, and it was decided to undertake the investigation, particularly as no detailed chemical examination seemed to have been made previously. The accounts of violet bacterial pigments met with in the literature are very fragmentary and somewhat confusing, and deal mainly with their absorption spectra. Lecoq de Boisbaudran [1882] extracted a violet pigment produced by a bacterium, which he does not describe, when grown on starch paste, and records its absorption spectrum. Similar colouring matters obtained from *B. violaceus* and *B. janthinus* are described by Schneider [1895]. The pigment from *B. violaceus* was submitted to a spectroscopic analysis by Hartley [1913], and was considered by him to differ from those described by the authors mentioned inasmuch as it showed more marked absorption in the red. It is possible, however, that the pigment in all these cases was the same—for the earlier spectroscopic observations of Lecoq de Boisbaudran and Schneider were probably not exact enough for the differences to be significant—while the qualitative chemical reactions ascribed to the pigment by all these authors are in fair agreement. The organism producing it is now commonly described as *Chromobacterium violaceum* (Bergey, *Manual of Determinative Bacteriology*). Hartley prepared the pigment by extraction of slices of potato, on which the bacterium grows well, with absolute alcohol, followed by evaporation to dryness. The material obtained in this way can hardly have been very pure, but probably sufficed for the qualitative tests employed and for spectroscopic examination in the absence of any strongly coloured impurities.

For the present work the pigment has been obtained mainly by growth in a lactose broth medium. Isolation and purification presented some difficulties as the colouring matter is very unstable in alkaline solution in which it dissolves readily, and is not very soluble in organic solvents. An analysis of the material purified as carefully as possible, in the circumstances, gave as a first approximation a formula of $(C_{10}H_{12}O_3N)_n$. Approximately one-fifth of the total nitrogen is removed by the action of nitrous acid, pointing to a value $n = 5$ (or some multiple of five). This was confirmed by a determination of the molecular weight of the substance by a modification of Rast's camphor method devised for the purpose. The value obtained, $M = 917$, agrees fairly well with that required for $n = 5$.

Preparation and isolation of the pigment.

In the earliest stage of the work sterilised skim-milk was used as a medium for growth as it possessed the advantages of being cheap and of permitting abundant pigment formation. The difficulties met with in filtering this medium and in isolating the pigment in pure form from such a complex mixture led to its eventual replacement by the simpler nutrient lactose broth, made up as follows:

5 g. lactose,
5 g. peptone,
3 g. beef extract,
1 litre of water.

Six litres of the sterile broth contained in a number of flasks were inoculated with a pure culture of *C. violaceum* and kept for about three weeks at a temperature of 20°. The pigment, which is insoluble in water, is found partly in flakes at the bottom of the flasks and partly distributed as very fine particles throughout the medium. It was dissolved out at first by repeated extractions with a mixture of alcohol and ether but this method, though suitable for skim-milk, proved unnecessarily wasteful for lactose broth. In the end it was found simplest to filter off the colouring matter by pouring the culture medium on to large filter papers. Filtration was slow but could not be improved by the use of suction, as the papers became clogged. A stream line filter was also tried but was not successful for the same reason. As soon as all the medium had run through, the residue on the paper was washed with a little water, and allowed to dry in the air. The filter papers were then cut up and extracted with absolute alcohol in a Soxhlet until no more colouring matter appeared to dissolve out. As this process usually took a long time—10 to 12 hours—a "vacuum" Soxhlet was used, to prevent possible decomposition of the pigment by prolonged heating, *i.e.* the extractor was provided with a very long Liebig condenser connected to the water-pump. The temperature of the boiling alcohol in contact with the dissolved pigment could thus be kept below 50° and practically no alcohol was lost. After the removal of as much colouring matter as possible in this way, the filter papers were further extracted by boiling for a few minutes with successive portions of fresh alcohol. These extracts were filtered, added to the Soxhlet extract, and the whole evaporated to small volume under reduced pressure. From this, the pigment was precipitated by the addition of 3–4 times its bulk of water, filtered under pressure, washed with water, dried on the pump and extracted several times with chloroform to remove traces of fatty substances, etc. The chloroform extract was always coloured greenish-brown, apparently by a second pigment present in small amounts. The residual pigment was now re-dissolved in alcohol, filtered, reprecipitated by addition of water, filtered off, dissolved again in alcohol and concentrated under reduced pressure until only a few cc. remained, and the greater part of the pigment had separated out. The solid was filtered off, dried in a vacuum desiccator and used for analyses and other estimations. A further supply of a presumably less pure material was obtained by the addition of water to the alcoholic filtrate. The total yield of pigment from 6 litres of lactose broth was about 60 mg.

Properties. The solid pigment is a purple-black amorphous substance which decomposes on heating without melting. It is insoluble in water, chloroform and other chlorinated solvents, light petroleum, carbon disulphide and acetic acid. It dissolves in ethyl alcohol to a beautiful bluish-violet solution, in ether (especially ether containing some alcohol). and in acetone to give purple solutions. Although these solutions are strongly coloured, the concentration of dissolved substance for saturation is low—probably not more than about 0·1 %. Pyridine—the best organic solvent observed—contained when saturated at ordinary temperature about 0·5 % of the pigment. Alkalis dissolved the pigment readily to green solutions, which, on heating, rapidly turned blood red, and then slowly passed through reddish brown shades to brown. Some structural change seems to occur on solution in alkali, as addition of acid does not restore the original colour.

Heating a pyridine solution of the pigment with acetic anhydride gave a red solution which was fairly stable to further heating. On the grounds that the pigment might contain a reactive NH_2 group, attempts were made to prepare derivatives by the action of formaldehyde and of toluene-*p*-sulphonyl chloride in pyridine solution respectively. No change was observable although in the latter case boiling was continued for several hours.

A number of tests were carried out on alcoholic solutions of the pigment. These appeared to be fairly stable to reducing agents, remaining unchanged when acted upon by sulphur dioxide, zinc and dilute sulphuric acid, and stannous chloride. Potassium permanganate instantly gave a brownish-yellow solution, while bromine water brought about immediate decoloration.

The action of nitrous acid on the pigment gives a reddish brown solution from which a flocculent brown precipitate, very difficult to filter, eventually settles out. After destruction of the excess of nitrous acid by urea and acidification with dilute sulphuric acid, the brown substance was extracted by *n*-butyl alcohol, washed free from acid with sodium bicarbonate solution, and isolated by removal of the alcohol. The product did not respond to the

Liebermann nitroso-reaction, so that the original pigment does not seem to possess a secondary amine group.

The pigment dissolves in concentrated nitric acid to give an orange solution which becomes more intense in colour on addition of alkali. It does not respond to Millon's test.

Analysis:

Found for pigment	Calc for $(C_{10}H_{12}O_3N)_n$.	Calc. for $C_{50}H_{59}O_{15}N_5$
C = 62·07 %	C = 61·86 %,	C = 61·92 %
C = 6·04 %	H = 6·18 %,	H = 6·09 %
N = 7·14 %	N = 7·22 %,	N = 7·23 %
O = 24·75 % (by diff.)	O = 24·74 %.	O = 24·76 %

From the content of amino-nitrogen and the molecular weight it appears (as will be seen later) that $n = 5$ which would make the formula for the substance $C_{50}H_{60}O_{15}N_5$. The slightly differing formula, $C_{50}H_{59}O_{15}N_5$ is suggested for the pigment as it is in somewhat better agreement with the analytical data.

Action of nitrous acid on the pigment.

An attempt was made to determine the nitrogen liberated from the pigment by the action of nitrous acid in the Van Slyke micro-apparatus. Several difficulties presented themselves. The only solvents in which the pigment was sufficiently soluble to permit the use of amounts even as great as 10 mg. were pyridine and sodium hydroxide solution. The use of the former seemed seriously to affect the method even when a sufficient excess of acetic acid was subsequently introduced to neutralise it, giving low results and much slower evolution of nitrogen when added to a known solution of tyrosine. An alkaline solution of the pigment had therefore to be introduced, although it was almost certain that permanent chemical changes had been produced in the substance by solution in alkali. It was furthermore obvious that if only about one-fifth of the nitrogen could be removed by the action of nitrous acid (as appeared to be the case), the volume of nitrogen obtained from about 10 mg. of pigment would not much exceed 0·2 cc. In these circumstances it was of the utmost importance to see that the blanks were as small as possible. Blank estimations were carried out with a number of samples of sodium nitrite and found, however, to be surprisingly large, in all cases amounting to about 0·5 cc. for an evolution of 25 cc. of nitric oxide. Some values obtained from a sample of Kahlbaum's nitrite were, *e.g.* 0·46, 0·46, 0·59, 0·44, 0·48, 0·45 cc. It was hoped that the gas found in the blanks might be nitrous oxide, or at least contain a definite percentage of nitrous oxide, so that a gas analysis might be used to determine in an actual estimation how much of the total volume was due to the blank and how much to the substance under examination, but an analysis of the "blank" gas in a Bone and Wheeler apparatus proved it to consist entirely of nitrogen. Recrystallisation of the sodium nitrite led to a slightly lower value for the blanks, which were, however, still considerable.

At this stage it was found that Van Slyke [1913] had already remarked on this matter as follows: "The substance in the nitrite which gives the small amount of gas obtained on blank determination we have never been able to identify or remove...." To test the point further we prepared a solution of nitrite by the method recommended by Divers [1899], namely by passing the gases obtained by heating arsenious acid with nitric acid into sodium carbonate solution. The blank from a sample of nitrite prepared in this way was 0·25 cc. for a nitric oxide evolution of 35 cc. In view of these facts it seems possible that part at least of the blank is due to a secondary decomposition of nitrous acid with formation of nitrogen, and consequently cannot be avoided in these determinations. The procedure which had to be adopted finally was to make a determination of amino-nitrogen and follow it immediately by a blank estimation, taking care that the volume of nitric oxide evolved in the two cases should be exactly the same. No great accuracy could be expected for the result in view of the relative proportions of blank and estimation, so that the agreement found for duplicates below is very satisfactory.

(*a*) 8·725 mg. pigment dissolved by warming with 3 cc. *N* NaOH and introduced into the reaction vessel.

Shaken, allowed to stand for a half-hour, and then shaken vigorously for five minutes.

Vol. of moist N = 0·66 cc.; vol. of blank N = 0·46 cc. Temp. 16° Bar. 770 mm.

$$\frac{\text{Amino-N}}{\text{Total N}} = 19\cdot0\ \%.$$

(*b*) 8·255 mg. pigment. Vol. of moist N = 0·352 cc. Blank = 0·17 cc. Temp. 16°. Bar. 767 mm.

$$\frac{\text{Amino-N}}{\text{Total N}} = 18\cdot2\ \%.$$

These values indicate that approximately one-fifth of the total nitrogen is removable by the action of nitrous acid.

Molecular weight. The first attempts to determine the molecular weight of the pigment were made with the Pregl ebullioscopic apparatus, using alcohol as solvent. Although the solution was very deeply coloured, no appreciable rise of boiling point could be detected on addition of the pigment on account of its low solubility and apparently high molecular weight. Acetone, and methyl alcohol, both of which dissolve the pigment to give highly coloured solutions, were also tried as solvents but with as little success. An attempt to use pyridine had to be abandoned on account of the impossibility of obtaining a steady temperature. Fortunately the pigment showed slight solubility in camphor, though only sufficient to give a freezing point depression of about 0·5° by the ordinary Rast method for molecular weight determination. A modification of this method was, however, devised by the authors (and is elsewhere described [1927]), which allowed of the accurate determination of molecular weights from such small depressions of freezing point. The following estimations have been made by its use.

(*a*) 3·77 mg. pigment in 434 mg. camphor. $\Delta = 0{\cdot}38^\circ$.
$M = 923$.
(*b*) 3·14 mg. pigment in 348 mg. camphor. $\Delta = 0{\cdot}40^\circ$.
$M = 910$. Mean value $M = 917$.
$(C_{10}H_{12}O_3N)_4$ requires $M = 776$.
$C_{50}H_{59}O_{15}N_5$ requires $M = 969$.

The molecular weights found point to the latter formula. Although the results are somewhat low, this is not unnatural in view of the very great influence of traces of simpler impurities on the freezing point, and the unavoidable inclusion in the weight of the solvent of camphor on the sides of the containing vessel.

SUMMARY.

The pigment produced by *Chromobacterium violaceum* has been isolated and examined. Determinations have been made of the empirical formula, molecular weight, and nitrogen removable by the action of nitrous acid. The formula $C_{50}H_{59}O_{15}N_5$ is provisionally suggested for the pigment.

The authors desire to express their thanks to M. Grimes, Ph.D., of the Dairy Bacteriology Department of this College, for assistance in the preparation of pure cultures, media, etc., and to the Chemical Society for a grant which partly defrayed the cost of this investigation.

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CXLI. THE PREPARATION OF TAURINE IN CONSIDERABLE QUANTITY.

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(*Received August 1st, 1927.*)

In order to carry out certain experiments to investigate the action of taurine in its physiological aspects it became necessary to prepare this compound on a considerable scale. Various possibilities of synthesising it were explored but it appeared that in all probability the cheapest and most expeditious method of obtaining the necessary quantity would be to prepare it from some animal product[1], and ox-bile was, under the circumstances, chosen as being most suitable.

Preliminary experiments with fresh ox-bile obtained from the slaughter house, in which the bile was boiled up for eight hours with one-third of its weight of concentrated hydrochloric acid and the product separated from glycine by treatment with alcohol according to the method of Hammarsten [1901] gave yields of 5 g. of taurine per litre of bile. Difficulties were, however, encountered when it was attempted to apply this method on a large scale. For instance it was difficult to obtain three hundred litres of bile from an ordinary slaughter house even over a considerable time, and it was therefore very useful to find that the commercial preparation of sodium tauroglycocholate which can be purchased in large quantities may be used instead of the fresh ox-bile, over which it has many advantages as a source of taurine[2]. It seems, therefore, desirable to put on record the results which have been obtained by the use of this material. Preliminary experiments showed that on a small scale yields of 3–4 g. taurine were obtained from 100 g. sodium tauroglycocholate. On the large scale it was found that 5 kg. of the sodium salt yielded 173 g. taurine. The details of the large scale process follow.

Experimental.

Sodium tauroglycocholate (5 kg.) is dissolved in water (20 l.) and concentrated hydrochloric acid (10 l. $d = 1{\cdot}18$) is added. The mixture is efficiently stirred and boiled under reflux for ten hours. On cooling, the aqueous solution is decanted from the solid hard mass of dyslysin and the latter washed well with water. The aqueous solution and washings are evaporated *in vacuo* to about 2 l., and whilst still hot filtered from the sodium chloride (A) which has separated. The filtrate is further concentrated to 300 cc. on the water-bath and again filtered whilst hot (B). The filtrate is diluted with $0{\cdot}5N$ HCl to 500 cc., poured into absolute alcohol (5 l.), and after standing overnight, the taurine which separates along with some sodium chloride is filtered off, whilst the glycine remains in solution. Recrystallisation of this material from an equal weight of boiling water yields almost pure taurine containing a barely detectable amount of sodium chloride. The compound is washed with absolute alcohol and the alcoholic washings mixed with the filtrate: this precipitates taurine and sodium chloride contained in the mother-liquors. The sodium chloride obtained at the stages A and B (mentioned above) is extracted with a small quantity (about 200 cc.) of boiling water and the solution is filtered hot, the process being repeated if necessary. These filtrates containing taurine, sodium chloride and possibly some glycine are diluted to twice their volume with $0{\cdot}5N$ hydrochloric acid and the solution is added to ten volumes of absolute alcohol so as to effect separation from the glycine. The precipitated taurine obtained is crystallised from hot water. The mother-liquors from all these crystallisations are evaporated down to a small volume, filtered hot from the sodium chloride which has separated and allowed to cool. The taurine is filtered off and if necessary again crystallised. The combined sodium chloride residues are extracted with boiling alcohol containing potassium hydroxide (2 %) and the hot alkaline extract is neutralised with acetic acid [cf. Abderhalden, 1909]. The comparatively small amount of taurine which separates on cooling is filtered off and purified by recrystallisation from an equal weight of hot water. The total yield of taurine from 5 kg. sodium tauroglycocholate amounts to 173 g.

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[1] Since the work described in this paper was carried out a synthetic method has been described by Marvel, Bailey and Sparberg [1927] which appears capable of giving good yields of taurine from comparatively cheap materials.

[2] It may be noted that in a paper by Rose and Huddleston [1926] in which the effect of taurine on the growth of animals deprived of cystine was investigated references are made to the use of desiccated bile as a source of taurine.

The alcohol used in the above purification is distilled off and the resulting aqueous solution is evaporated to dryness on the water-bath. The residue is extracted with boiling absolute alcohol, filtered hot, and the filtrate saturated with dry hydrogen chloride. On standing in the ice-chest overnight crystals of glycine ester hydrochloride separate. These are filtered off, washed with a little alcohol, and dried. The yield is 104 g.

The hard mass of dyslysin, which remains after hydrolysing the sodium tauroglycocholate with hydrochloric acid, is not easily attacked by aqueous sodium hydroxide solution but it is soluble in boiling alcoholic potassium hydroxide solution. When the alcohol is removed by distillation in steam an aqueous solution remains from which the bile acids may be precipitated by means of mineral acid. It may be here noted that attempts were made to obtain taurine by alkaline hydrolysis of sodium tauroglycocholate as described by Abderhalden [1909], but on a large scale the considerable quantities of alkali and acid, which must be used, result in the formation of a very large amount of salt and the difficulty of isolating taurine in good yield and in pure condition becomes very great.

The authors desire to express their thanks to Sir James Walker, and the technical staff of the Chemistry Department, Edinburgh University, for assistance in carrying out some of the major operations in connection with this research, and to the Department of Scientific and Industrial Research for a personal grant to one of them (R. H. S.).

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CXLII. CARBON MONOXIDE AS A TISSUE POISON.

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(*Received August 2nd, 1927.*)

Carbon monoxide is relatively harmless to invertebrates, micro-organisms and plants. Thus Frankland [1888], Linosier [1889] and others, who found that very large amounts inhibited plant and bacterial growth, did not show conclusively that CO is any more poisonous than N_2. J. S. Haldane [1895] showed that cockroaches would live for long periods in a mixture of 80 % CO and 20 % O_2. Warburg [1926] however found that when the proportion of CO to O_2 in a gas mixture was raised to about 5, the O_2 consumption of yeasts and a coccus was diminished. He also showed that strong light neutralised the effect of CO. He concluded that CO combined with a catalyst in the cells, with which O_2 must combine before it can oxidise other substances, and that this catalyst was probably an iron compound analogous to haemoglobin. As the amount of CO needed to cause a given drop in the O_2 consumption is roughly proportional to the partial pressure of O_2, the analogy with haemoglobin can be carried further.

In the experiments here described Warburg's observation is extended to moths, seeds, and rats. Owing to the difficulty of obtaining a standard of respiration the general behaviour of the organism and not its O_2 consumption was observed. Wax moths (*Galleria mellonella*) from a culture kindly put at my disposal by Dr Keilin, were placed in a glass tube through which mixtures containing various amounts of O_2 and CO were passed. The tube containing the moths was kept in a water-bath at 20°, and the gas passed through sampling tubes before and after reaching the animals. Their contents were analysed after each experiment. If the quantities of O_2 differed by as much as 0·2 % the experiment was rejected. The CO content of one or both tubes was also determined by combustion.

In low percentages of O_2 the moths rapidly became sluggish in their movements, fell to the bottom of the tube, and after two or three minutes became immobile even when it was tapped. On readmitting air, even after an hour or more, they recovered completely. In larger but still inadequate amounts of O_2 various degrees of weakness were observed, ranging from inability to fly or climb to immobility save when stimulated. The symptoms were qualitatively the same whether they were due to O_2 want or to a combination of this with CO poisoning. There was little difference of susceptibility between individuals, but in order to allow for this three moths were always used, and the behaviour of the majority recorded. Such differences occurred in twelve out of the forty-nine experiments on which Fig. 1 is based. Although various degrees of ataxia could be distinguished, behaviour is classified according as the moths were normal, ataxic, or wholly immobile even on stimulation. Six points recording experiments in the absence of CO have been omitted, as have the results of several experiments done under less rigid conditions which were, however, confirmatory.

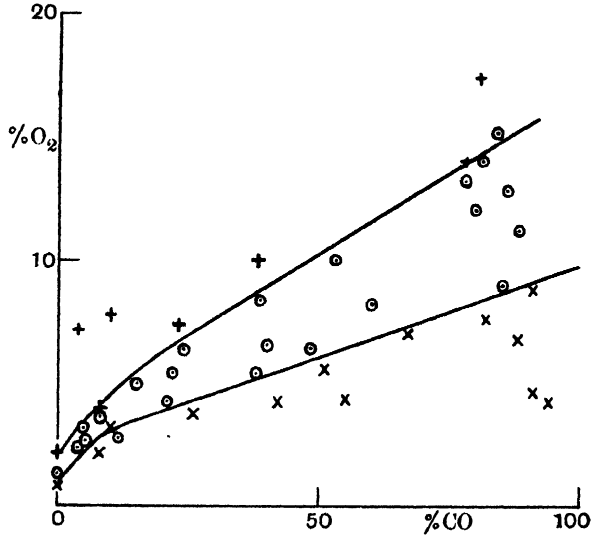

Fig. 1.

+ Moths normal. ⊙ Moths ataxic. × Moths immobile.

In the absence of CO the moths were motionless in mixtures containing less than 1·0 % O_2, normal in over 2·0 %. In presence of 80 % CO about 8·4 % O_2 is needed for mobility and 14 % for normal behaviour, while in intermediate concentrations of CO intermediate amounts of O_2 are required. The lines separating the three areas in the diagram are straight when the CO exceeds 30 % but bend downwards quite definitely at low percentages. The experiments were done in diffuse daylight or electric light, but no definite effect was produced by the proximity of a powerful lamp, probably because the moths are opaque.

The germination of cress (*Lepidium sativum*) seeds in various gas mixtures was next studied. The seeds were placed on wet sand in each of a series of bottles containing known gas mixtures, and their appearance noted daily. Since a preliminary series, which on the whole agreed with later results, showed evidence of leakage in one bottle, the bottles were immersed in a water-bath at room temperature in partial darkness. Two series of twelve bottles each made up in February and March gave quite consistent results. Thus in the second series in the absence of CO the majority of seeds had split in three days in 8·9 % O_2, in three and a half in 6·9 %, in four in 4·7 %, in eight in 3·0 %, while none split in sixteen days in 2·1 % or 1·4 % though all germinated on admitting air. In the presence of 70 % to 87 % of CO all had split in two and a half days in 19·2 % O_2, in three in 15·3 %, in five in 10·7 %, but after sixteen days only a few had split in 6·2 % and none in 4·1 % or 2·6 %. All germinated on admitting air. By comparing the amounts of germination and growth it was possible to equate the effects of different gas mixtures. It was found that for equally rapid germination and subsequent growth between two and three times as much O_2 was needed in the presence of about 80 % CO as in its absence. No qualitative difference was noted between the effects of CO and of simple O_2 want. When the experiment was repeated in the summer on a smaller scale no change was noted in the susceptibility to O_2 want, but that to CO was somewhat increased, not all the seeds having split after five days in 83 % CO + 11·8 % O_2. This may have been due to the higher temperature or to ageing of the seeds.

The experiments on rats were, of course, complicated by the presence of haemoglobin. Advantage was therefore taken of the fact that mammals can live on O_2 dissolved in their blood under high pressure even when almost all their haemoglobin is combined with CO [Haldane, J. S. 1895]. A small iron chamber was fitted with rubber washers so that its door could be made air-tight, a window for observation, and three taps, two of which were connected with cylinders containing O_2 and CO under pressure; the third could be opened to the outside. When a rat was placed in three atmospheres of O_2 and one of CO its behaviour was almost normal, but in some cases the movements of the hind limbs were rather clumsy. In two atmospheres of O_2 and one or half an atmosphere of CO movement was difficult, but there was little increase of breathing and no convulsions.

There are no exact data as to the affinities of rat's haemoglobin for CO and O_2 at body temperature. At 15° the ratio is about 280 : 1, that for a human haemoglobin being 400 : 1 [Anson, Barcroft, Mirsky and Oinuma, 1924], whereas at 37° the human value is about 250 : 1. If then the temperature coefficient of the equilibrium $HbO_2 + CO \rightleftharpoons HbCO + O_2$ is the same in rats and men, the rat's ratio at body temperature is about 175 : 1. Hence at three atmospheres pressure of O_2 and one of CO the rat's haemoglobin was about 98·3 % saturated with CO, the remaining 1·7 % being a very inefficient carrier of O_2 [Haldane, J. S., 1912]. Since a single atmosphere of CO is sufficient to nullify practically all the haemoglobin, the effects of any further addition of that gas can only be on the tissues, since, as will be seen below, the addition of a physiologically inert gas such as N_2 is without effect.

The following experiments, all on the same rat H, are typical.

July 9th

3.50 Pressure in chamber raised to total of 3·1 atmospheres of O_2

3.52 1 atmosphere CO added. Rat at first slightly excited, perhaps by the smell.

4.22 Rat normal. Excess of pressure over atmospheric has fallen from 48 lb. per sq. in. to 43. Lower pressure to 15 lb. Raise to 45 lb. with O_2.

July 9th	
4.25–4.38	Pressure repeatedly lowered and then raised with O_2 until the partial pressure of CO was reduced to 0·13 %. The rat was then taken out, and was unaffected.
July 11th	
3.2	Pressure raised to 3·1 atmospheres O_2.
3.4	1 atmosphere CO added.
3.6	2 atmospheres N_2 added.
3.30	Pressure fallen from 77 to 64 lb. Rat normal. Pressure lowered and CO diluted as above.
4.0	Rat removed from chamber, quite normal.
July 13th	
4.55	Pressure raised to 3·1 atmospheres O_2.
4.57	Add 1 atmosphere CO. Rat normal.
5.0	Add second atmosphere CO.
5.1	Deep and rapid breathing.
5.2	Breathing rapid. Rat goes into violent convulsions.

Although the O_2 pressure was subsequently raised to 6 atmospheres the rat's breathing became shallow and it died.

Altogether eight experiments of this type were made. In all cases the addition of sufficient CO caused hyperpnoea and convulsions. If O_2 were at once added and the pressure quickly lowered so as to dilute the CO it was generally possible to save the animal's life. The rats used showed very different susceptibilities to high pressures of CO, all from the same cylinder. Thus while rat H died on exposure to 3 atmospheres of O_2 and 2 of CO, and rat F in 2 of O_2 and 2 of CO, rat B appeared practically normal after four minutes in this mixture, though it died on raising the CO pressure to 3 atmospheres. Finally rat D appeared little affected by four minutes' exposure to 2 atmospheres of O_2 and 3 of CO, though killed by an additional atmosphere of CO. These differences may of course be due to different relative affinities of the catalyst for CO and O_2, but it seems equally plausible to trace them to differences in the cerebral vaso-constriction caused by O_2 [Dautrebande and Haldane, J. S., 1921; Tinel, 1927]. No definite evidence was obtained that poisoning by CO at high pressure can be relieved by high pressures of O_2, but attempts to prove this were rendered difficult by the fact that convulsions due to acute O_2 poisoning set in at about 7 atmospheres pressure of O_2. It is, however, clear that CO has a poisonous action on rats apart from its combination with haemoglobin.

In order to test the possibility that the effects observed were due to impurities in the CO, the gas was made by two different methods. Most of the experiments were made with gas prepared by heating formic and sulphuric acids, and passing the gas evolved through soda or over soda-lime. The analytical figures obtained on combustion suggested that it contained about 2 % H_2. A sample prepared from oxalic and sulphuric acids had indistinguishable physiological properties. The point in Fig. 1 representing a mixture containing 67·1 % CO and 6·99 % O_2 was obtained with this gas. Two of the moths were quite immobile in it, one preserved reflex movements on tapping. Hence, as was to be expected, the mixture was just on the border-line of those producing immobility and ataxia.

All samples of CO had a definite smell generally compared to that of garlic, but by one colleague to that of tar. This smell was not appreciably diminished by passing the gas over adsorbent charcoal or through bromine water and soda solution, which might be expected to remove large or unsaturated molecules. It is therefore likely that odour is a physiological property of CO [Haldane, J. S., 1921]. It is only observed in fairly high concentrations, which may of course be smelt with safety provided the operation is not repeated too often at short intervals.

DISCUSSION.

The experiments of Keilin [1927] on extracts of yeast, mammalian muscle, oatmeal, and potatoes, make it fairly clear that CO acts by combining with polyphenol or indophenol oxidase, or at least some component of that oxidase system. Since the amount of CO needed to produce a given degree of inhibition depends on the O_2 pressure it follows that both combine with it in the same way. Thus in the case of yeast saccharase, fructose competes with sucrose for the enzyme molecule, and therefore depresses hydrolysis more in weak than in strong sucrose solutions, whereas glycerol and other substances which do not so compete with sucrose depress hydrolysis to a degree nearly independent of the sucrose concentration [Michaelis and Pechstein, 1914]. In this respect CO is clearly comparable with fructose rather than glycerol. In view of the results of Keilin and the great speed with which O_2 must diffuse within a cell it is very unlikely that the substance sensitive to CO is a mere carrier of molecular O_2 like haemoglobin.

If CO combines with a catalyst X normally concerned in oxygen activation, the following reactions may be supposed to occur:

$$X + O_2 \rightleftarrows XO_2$$
$$XO_2 + 2A \rightarrow X + 2AO$$
$$X + CO \rightleftarrows XCO$$

where A is an oxygen acceptor, possibly 2 H atoms from an activated molecule. Let x be the concentration of CO, y that of O_2, $e - p - q$ that of X, p that of XO_2, q that of XCO and a that of acceptor. Let k_1, k_2 be the velocity constants of the first reaction, k_3 of the second, k_4, k_5 of the third. Then when the reactions are proceeding steadily or in equilibrium, as the case may be, we have [Briggs and Haldane, J. B. S., 1925]

$$k_1 (e - p - q)\, y = (k_2 + k_3 a^2) p, \quad k_4 (e - p - q)\, x = k_5 q.$$

Eliminating q we find

$$p = \frac{e}{1 + \left(\frac{k_2 + k_3 a^2}{k_1 y}\right)\left(1 + \frac{k_4 x}{k_5}\right)}.$$

Hence the velocity of oxidation,

$$v = k_3 a^2 p = \frac{k_3 a^2 e}{1 + \left(\frac{k_2 + k_3 a^2}{k_1 y}\right)\left(1 + \frac{k_4 x}{k_5}\right)}.$$

If V be the velocity when the O_2 concentration is so high that v no longer depends on it, and if

$$K_{O_2} = \frac{k_2 + k_3 a^2}{k_1},$$

i.e. the Michaelis constant of the oxygen activator,

$$K_{CO} = \frac{k_5}{k_4},$$

i.e. the equilibrium constant of the third reaction,

$$\therefore v = \frac{Vy}{y + K_{O_2}\left(1 + \frac{x}{K_{CO}}\right)}. \quad \text{.........................(1)}$$

$$\left.\begin{aligned} \therefore \left(\frac{V}{v} - 1\right) y &= K_{O_2} + \frac{K_{O_2}}{K_{CO}} x, \\ \text{or,} \quad x + K_{CO} &= \frac{K_{CO}}{K_{O_2}}\left(\frac{V}{v} - 1\right) y. \end{aligned}\right\} \quad \text{.........................(2)}$$

Now if we suppose that a given degree of asphyxia as measured by behaviour or growth corresponds to a particular degree of reduction in the velocity of oxidation in certain cells, equation (2) should give the relation between the partial pressures of CO and O_2 required to produce it. For with a given degree of asphyxia a and hence K_{O_2} are presumably constant, though they may increase with increasing asphyxia.

Warburg showed that equation (1) did not hold for yeast, but that the initial effect of CO in diminishing oxidation was relatively less than the effect of larger amounts. This might have been due, among other things, to the existence of a component of the total oxidation not affected by CO, or to an increase with increasing asphyxia in the value of a, *i.e.* the amount of oxygen acceptors. Neither of these causes however would cause a deviation from the linear equation (2) such as was found in the case of *Galleria*. The relation however is nearly linear for large values of x, the equations for the two linear parts being $x + 25{\cdot}0 = 6{\cdot}9y$ and $x + 28{\cdot}9 = 13{\cdot}2y$. The agreement in the two values, 25 % and 29 % for the value of K_{CO} is as good as could be desired. Hence the catalyst behaves as if it were half saturated with CO in the absence of O_2 at a pressure of about 27 % of an atmosphere.

The deviation from linearity in low concentrations of CO may be due to several causes. In the first place, the dissociation curves of XO_2 and XCO may deviate from rectangular hyperbolae as do those of the corresponding haemoglobin derivatives. But since the presence of small amounts of CO in a gas mixture containing only enough O_2 partially to saturate the haemoglobin probably facilitates the formation of oxyhaemoglobin [Douglas, Haldane, J. S., and Haldane, J. B. S., 1911] such deviations from the law of mass action as might account for this effect would be in the opposite direction to those of haemoglobin.

It seems at least equally probable that the cells of the moth contain several catalysts of the type considered, having different affinities for CO and O_2, only one however being the limiting factor to oxidation above a certain partial pressure of CO. This possibility is borne out by the very different effects of CO on moths and cress. In 80 % CO the moth needs 7 or 8 times as much O_2 as when the O_2 is diluted with N_2, the seed only about 2½ times. It is very doubtful whether the difference of about 8° in the temperature will account for so large a difference, which is probably analogous to that between different haemoglobins. If the catalyst differs markedly in different cells of the same organism it is likely that measurements of O_2 consumption would yield a less simple result than qualitative experiments depending on a relatively small number of cells in the nervous system.

The actual O_2 pressures in the tissues during these experiments are of course unknown. Those of CO must be the same as in the gas mixture. As however for a given degree of asphyxia the rate of oxidation in the tissues is presumably constant, the O_2 pressures in the gas mixture must, if the O_2 reaches the tissues by diffusion, exceed that in the tissues by a constant amount, which must be less than 2 % of an atmosphere for the barely ataxic moths, less than 1 % for the barely immobile. The effect of allowing for this difference would be to lower the two curves in the figure by a small amount, constant for each curve. Calculations based on their upper portions would barely be affected, nor would such a correction account for their curvature.

The experiments on rats show that a CO-sensitive substance exists in the mammalian brain, as well as in the muscles where Keilin found it. Presumably it is of the same nature as the muscle catalyst, and hence as that in invertebrates and plants. If so CO, like cyanide, stimulates the respiratory centres by inhibiting oxidation there. The possibility that they are made sensitive to oxygen by a substance such as haemoglobin which changes its properties with the partial pressure of O_2 is rendered very unlikely.

The nature of the catalyst is of course unknown, though it may well be an iron-porphyrin derivative. Keilin's results show that it is a component of the polyphenol oxidase system, but it does not of course follow that the oxidation of polyphenols or related substances is a stage in every oxidative process inhibited by CO. Oxygen activated by the catalyst in question may be required for the oxidation of a variety of substances. As such catalysts have now been found in bacteria, yeasts, higher plants, insects and mammals, they are presumably present in the majority, if not all, of aerobic organisms.

SUMMARY.

1. The movements of a moth and the germination of cress seed are inhibited by CO. The greater the partial pressure of O_2, the more CO is required.

2. Rats living on O_2 dissolved in their blood in presence of sufficient CO to combine with almost all their haemoglobin are killed by the addition of more CO, which must affect some substance in their tissues.

3. It is concluded that cells contain a catalyst of oxidation which is poisoned by CO. Its affinity for CO is determined in the case of the moth. Its affinities differ in different species, and perhaps in different tissues.

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CXLIII. GROWTH AND REPRODUCTION ON SYNTHETIC DIETS. II.

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In an earlier paper [Hartwell, 1926] a description was given of various synthetic diets which produced good but not maximal growth in young rats. The importance of a really good synthetic diet for use as a standard in nutrition experiments is self evident, and certain modifications of the foods used previously [Hartwell, 1926] have yielded satisfactory results, although, as yet, no diet has been found which will produce growth equal in rate to that obtained on kitchen scraps. The improvement in reproduction and lactation on these modified diets is even more marked than with growth, and therefore it is thought advisable to publish these results briefly in that they may be of use to others working on similar lines.

The difficulty of obtaining normal reproduction on synthetic diets is commented on by many researchers, while the actual rearing of the young when the mothers are living on such foods is almost impossible. Nelson, Jones, Heller, Parks and Fulmer [1926] write that "one thing is certain, that reproduction is not normal on certain synthetic diets and diets involving restricted food materials." They find that the number of young born may be comparatively few and that the majority die before weaning, although they have carried their experiments through several generations.

Using a basal synthetic diet of caseinogen, salt mixture, butter fat and dextrin, with wheat, rye, barley or corn as sources of vitamin B, Guest, Nelson, Parks and Fulmer [1926] found that rats reproduced normally, but that there was a high mortality among the young, due in their opinion to failure of milk secretion. Hogan and Harshaw [1924] reared four generations on a synthetic diet, but found that the number of litters was subnormal, the mortality of the young high and that any young which survived grew very slowly during the suckling period. Anderegg [1924] found a high mortality among the young even when the protein was given as whole milk powder, and Murphy and Jones [1926] state that on a diet of bran, salts, crisco and starch the rats produced offspring, but had little success in rearing them. Kennedy and Palmer [1926] used crisco instead of lard in the basal diet shown by Evans and Bishop [1923, 1] to be inadequate for reproduction, and obtained litters in three successive generations of rats. They found, however, that the mother rats had great difficulty in rearing their young even when the diet of each rat was supplemented by 15 cc. of whole milk throughout the lactation period.

Thus the failure to rear their sucklings when the mother is fed entirely on a synthetic diet appears to be universally recognised.

EXPERIMENTAL.

The preparation of the food, weighing the animals, etc., has been described previously [Hartwell, 1926]. The composition of the diets is given in the following table.

	Diets				
	A	*E*	*CE*	*CL*	*CLO*
Caseinogen	20 g.	16 g.	8 g.	14 g.	20 g.
Egg-albumin	—	—	8 g.	—	—
Lactalbumin	—	—	—	6 g.	—
Potato starch	64 g.	64 g.	64 g.	60 g.	64 g.
Butter	12 g.	16 g.	16 g.	16 g.	—
Cod-liver oil	—	4 cc.*	4 cc.†	4 cc.	14 cc.
Salt mixture (Hartwell)	4 g.	4 g.	4 g.	4 g.	4 g.
Marmite‡	5 g.	5 g.	5 g.	5 g.	5 g.
Water	300 cc.	300 cc.	300 cc.	300 cc.	300 cc.

* After first six weeks of experiment.

† After first four weeks of experiment.

‡ During lactation 10 g. of marmite were used instead of 5.

Three generations of rats were reared on diet *A*, but slight alterations in the fat were made from time to time. It was noticed that a few of the young rats developed slightly sore eyes at about 17–18 days of age and as this was first seen in the winter it was thought advisable to increase the butter to 16 g. and decrease the starch to 60 g., thus keeping the percentage composition of the other dietary constituents constant. The increase in butter caused no improvement in the eye condition of the suckling young and therefore 4 cc. of cod-liver oil were added to the diet. This alteration, apparently, led to disastrous results in that the mother frequently died when the young were about to be born (this is described later in the paper) and so the cod-liver oil was omitted from the diet. The duration of the experiment and the alterations in diet are given below.

	Duration of experiment	Alterations in diet
1st generation	June 15, 1926–April 5, 1927	4 cc. cod-liver oil added to diet from March 5, 1927
2nd generation, from first litters	Sept. 9, 1926–March 17, 1927	Butter increased to 16 g. on Feb. 3, and 4 cc. cod-liver oil added March 4
2nd generation, from second litters	Oct. 12, 1926–March 16, 1927	Butter increased to 16 g. on Feb. 3, and 4 cc. cod-liver oil added March 4
3rd generation	Feb. 3, 1927–July 15, 1927	Expt. started with 16 g. butter, March 4 4 cc. cod-liver oil were added; May 4 cod-liver oil discontinued and butter reduced to 12 g.

RESULTS AND DISCUSSION.

All the rats fed on the synthetic diets described in this paper were in good condition throughout the experiments. The animals were firm to feel and their muscles were fit; there were no signs of general flabbiness, and except that the rats were slightly smaller at any given age, they could not be distinguished from stock animals fed on kitchen scraps supplemented by bread and milk.

Growth.

The rate of growth on diets *A*, *E*, *CE*, and *CL* was good, though not equal to that of stock animals fed on the kitchen scrap mixture (see Fig. 1). Diets *E*, *CE*, and *CL* produced slightly quicker growth than diet *A*. It is impossible to say what was the cause of this improvement since several factors were varied in each diet. It seems unlikely that the differences in the protein ration are of primary importance here, because diet *E* containing only 16 % of caseinogen produced better growth than diet *A* with 20 % of this protein,

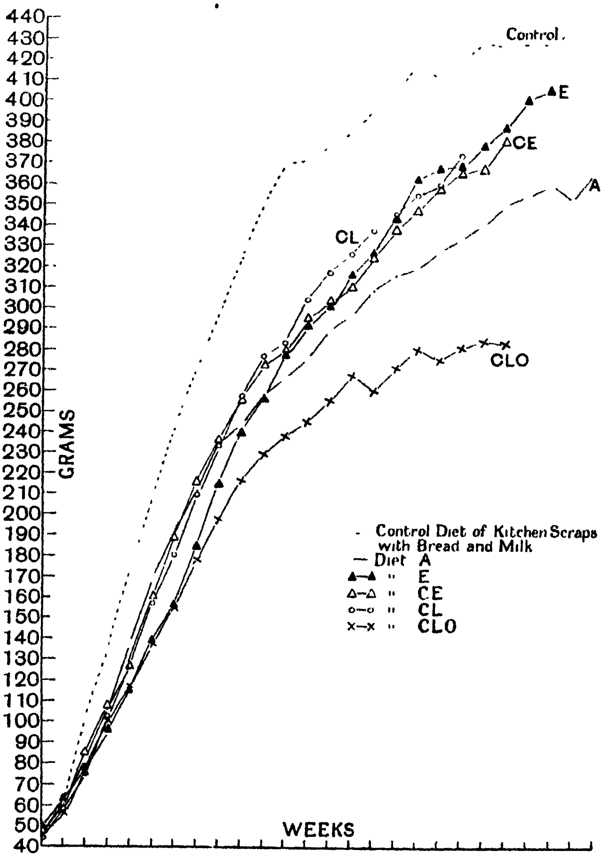

Fig. 1. Growth curves of male rats. Each curve is taken from the average of six animals.

and diets *CE* and *CL* containing two proteins at the level of 20 % produced no better growth than diet *E* with 16 % of one protein. The main difference between diets *E*, *CE*, and *CL* on the one hand and diet *A* on the other is the increased fat content of the former diets, diet *A* containing 12 % of butter, while diets *E*, *CE* and *CL* had not only an increase in butter to 16 % but also 4 cc. of cod-liver oil as well. This involves not only an increase of fat, but also of vitamins A and D in the diet, and possibly vitamin E.

Diet *CLO* gave comparatively poor results for growth, and the curve flattened early as seen in Fig. 1; the rats, however, were in good condition throughout the experiment. Diets *A* and *CLO* are similar in all respects except the fat, the former containing 12 % of butter and the latter 14 cc. of cod-liver oil in place of the 12 g. of butter. It is extraordinary that diet *CLO* produced such poor growth for it *must* have been adequate as regards vitamins A and D, and 5 g. of marmite were used while 2 g. have been found to produce good growth in young rats. It may be that there is an excess of vitamins A and D in this diet and that the poorer growth is due to upsetting the balance of the dietary constituents, or that vitamin E, stated by Evans and Bishop [1923, 1, 2] and Sure [1927] to be deficient, or present in only small quantities, in cod-liver oil, is essential for growth as well as for reproduction. If vitamin E can be stored in the animal body as can vitamin A, the good growth produced in the first few weeks of the experiment and the early flattening of the curve are easily understood.

Reproduction.

(*a*) *Diet CLO.* The fat in this diet was cod-liver oil, which it was hoped to use in place of butter in synthetic diets, in order to have a constant supply of vitamins A and D throughout the experiments, it having been shown by Luce [1924] that the antirachitic value of cow's milk varies with the season of the year. The six does in this experiment produced no litters, though the feeding was continued for 21 weeks, during which time other rats on synthetic diets would each have had two or three families. *Post mortem* examinations showed abnormal uteri and mammary glands, the histological details of which will be published later. The males varied, some appearing almost normal, while others had testes of very low weight. When it was found that this variation occurred, two males with apparently well formed testes were kept for a further period and mated with stock does which had previously reared litters, but no families were born. This experiment appears to confirm the work of Evans and Bishop [1923, 1] that cod-liver oil does not contain vitamin E, regarded by these workers as essential for reproduction. It should however be pointed out that the methods of investigation are not similar; Evans and Bishop used what might be termed a "curative method," while the experiments described in this paper are of the "preventive" type. The assumed absence of a special reproductive dietary factor is a very obvious explanation of these results, but it must be recognised that in both series of experiments the diets contained large amounts of cod-liver oil and therefore it is possible that an upset of the balance of the various dietary constituents may be a factor of importance. Evans and Bishop [1923, 1] used as much as 24 % of cod-liver oil in one experiment, and they considered that one sample of their oil was at least eleven times as rich in vitamin A as the milk fat of their standard diet.

(*b*) *Diet E.* All six does became pregnant at the normal time, but no young were reared. One doe was killed because of a cancerous growth; three does had first litters either born dead, or the young were eaten immediately after birth, and all three died with their second litters; the other two does died with their first litters. The does appeared perfectly fit and well throughout gestation, but they became comatose and eventually died with the young unborn. Before death they frequently had brief convulsions at intervals during the comatose period. The mothers apparently died from internal haemorrhage; their paws and tongue were quite white, their muscles very anaemic and the liver appeared bloodless. The young seemed normal in all respects. Some of the animals were killed while in the comatose condition, so that the *post mortem* observations should be of more value. As a rule the rats died about two days after the young were due to be born. (This statement is based on general observation of pregnant does; over 2700 litters have been born during the author's research, and by experience it is easy to tell when to expect the birth of the young.) When the six does all died, five other stock females, which had previously reared litters, were put with the males. Four of these had litters, born normally, and three of them reared some of their young.

(*c*) *Diet CE.* This diet gave better results in that some litters were reared, but three out of the six does died, showing the same symptoms as described under diet *E*.

Details of the breeding are as follows:

Number of litter	Number of young born	Average weight in g. day after birth	Number reared	Average weight in g. at weaning (21 days)
Rat 1				
—	5 dead			
2624	7 + 2 dead	5·3	4	31·5
2679	8	6·25	5	30·4
Rat 2				
—	Born dead, rat died the next day			
Rat 3				
—	5. Eaten second day			
—	Born dead			
Rat 4				
—	Died at birth of first litter			
Rat 5				
2589	3. Dead second day.			
2642	1 + 1 dead	4. Eaten third day		
2680	4 + 3 dead	6	4	28·75
Rat 6				
2590	7	6·3	5	30·6
—	Died when litter born			

(*d*) *Diet CL.* The results of this experiment are very similar to those obtained with diet *CE*, three does dying at, or soon after, the birth of a litter.

The few litters born to animals fed on diets *CE* and *CL* compared with those born to rats on diet *A* must not be taken as evidence that the diets *CE* and *CL* are less good for reproduction because these two experiments were of much shorter duration, each being discontinued after a third doe had died.

Number of litter	Number of young born	Average weight in g. day after birth	Number reared	Average weight in g. at weaning (21 days)
Rat 1				
2643	8 + 2 dead	4·6	3	23
2702	9	6	5	42
Rat 2				
—	Died with first litter			
Rat 3				
2635	2 + 1 dead	5. Eaten sixth day		
2677	4 + 2 dead	4·75	2	23·5
Rat 4				
2673	8	5·5	4	29·75
Rat 5				
2638	8	5	5	29·2
—	Died at birth of litter			
Rat 6				
—	Born dead, rat died a few days after			

(*e*) *Diet A*. This diet was by far the most satisfactory for reproduction and three generations were successfully reared. The rate of growth of the second and third generations was slightly slower than that of the first (see Figs. 2 and 3), but all the rats were in good health throughout the experiment and no dietetic disturbances, such as the hair falling out, etc., were ever seen. The slower growth of the second generation is in agreement with the results of Evans and Bishop [1923, 1]. The does in all three generations were fertile and litters were produced as frequently as in animals fed on a mixed diet of kitchen scraps. Two cages of second generation animals were kept, one from first litters and one from second; it was thought possible that if the diet were lacking in any way the second litters might show some effects not found in the first litters, which might have had the advantage of any reserve stores of the mother. However, no such differences appeared, and the two cages of rats were similar in all respects. The rearing of the young was by no means up to standard, although those which survived were usually fit and healthy, but below average weight at weaning. The first generation does reared 85 young out of a possible 156 (all litters were reduced to six on the day after birth); the second generation does from first litters reared 51 out of 100, the second generation from second litters brought up 30 out of 57 and the third generation reared 25 out of 52. This is a great improvement on the results obtained with synthetic diet 1 [Hartwell, 1926] on which only 10 young were reared out of 59. The number of young born to the does on synthetic diet *A* was not up to the controls, 10 being the largest litter, while stock animals produce as many as 18. There was little difference between the numbers born to the first and second generation rats, but there was a considerable drop in numbers per litter of the third generation families.

Details of the reproduction in the first generation are given in the following table:

Number of litter	Number of young born	Average weight in g. day after birth	Number reared	Average weight in g. at weaning (21 days)
Rat 1				
2348	6 + 3 dead	6	3	22·3
2388	6	5·7	3	30
2442	8	5·7	5	31·4
2483	3	5·6	2	30·5
2527	7	6	6	29·7
—	5. Born dead			
Rat 2				
2347	5	6. Eaten 15th day		
2383	5	5	2	14·5
2432	4	5	4	25·8
2472	1 + 2 dead	6. Eaten 5th day		
2498	3	5	3	24
2561	2	5·5. Eaten 6th day		
Rat 3				
2352	2	4. Eaten 5th day		
2382	7	5·7	5	28
2437	7	5·7	5	27·6
2475	5	4·2. Eaten 4th day		
2566	4	4·3. Eaten 6th day		
2587	4	4·5. Eaten 19th day		
Rat 4				
2345	3	4·6. Eaten 4th day		
2377	10	5·5	5	35
2406	7	6	6	29·3
2453	4	6·3	4	34
2500	7	5·4	6	29·3
Rat 5				
2346	5	5·6. Eaten 14th day		
2381	9	5·4	5	26·6
2430	4	5	1	38
2470	4	5·3	2	26·5
2514	3. Eaten 2nd day			
2555	1	6. Eaten 5th day		
2580	5	6	5	28·4
Rat 6				
2351	7	5·2	3	29·3
2396	4	6. Eaten 6th day		
2426	7	6·2	6	27·8
2469	4	5·5. Eaten 8th day		
—	Eaten at birth			
2524	6	6	4	30·8
2579	3	5·3	2	21·5

The results of breeding in the second and third generations are not given in detail, but the main points are shown in this table:

	Duration of expt. in weeks	Number of does	Number of litters born	Weight of young on second day (g.)		Average weight of young at weaning (g.) (21 days)
				Lowest	Highest	
1st generation	63	6	37	4·2	6·5	28·4
2nd generation						
From first litters	27	9	28	4·6	6·3	29·1
From second litters	23	7	15	4·7	6·3	26·9
3rd generation	23	8*	15	4·7	6·3	32·5

* Two of these does died at the birth of their first litters.

It is interesting to note that at 21 days the average weight of 87 young from the first generation is 28·4 g., while the corresponding figure from 81 young from the second generation is 28·3 g.

When an experiment was finished a *post mortem* examination was made on each parent animal, and from this examination, all organs appeared to be in a normal condition. This is especially interesting in connection with the third generation rats, where evidence of any dietary deficiencies might have been more marked. The third generation animals had very thick and strong abdominal muscles, the difference being as marked as in the wild rabbit compared with the hutch animal.

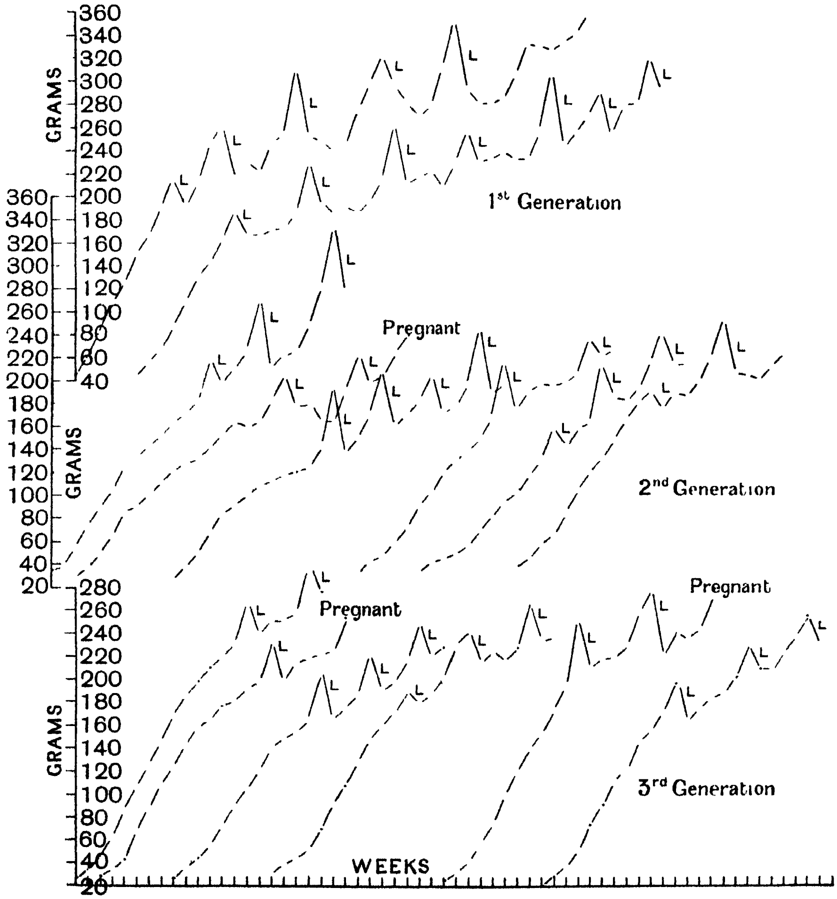

Fig. 2. Growth curves of some of the female rats fed on diet *A*. *L* = litter born.

One of the most striking features of these experiments is the death of the mother rat coincident with the failure of the young to be born. This appears to have a direct connection with the alteration of the fat content of the diet. With diet *A*, the first generation rats were receiving 12 g. of butter and each doe had 5, 6 or 7 litters, all born normally. In the second generation the fat was altered, but near the end of the experiment, and no catastrophes occurred. The third generation were started from weaning on the increased fat, *i.e.* 16 g. of butter and 4 cc. of cod-liver oil and two does died with their first litters. Consequently the fat ration was immediately changed to 12 g. butter and no more does died. With diets *E*, *CE* and *CL* the fat consisted of 16 g. of butter and 4 cc. cod-liver oil, and 12 of the 18 does died. An experiment is now in progress with a diet similar in composition to diet *A* but with 20 g. butter and no cod-liver oil, to see if the oil is the primary factor or if the actual amount of fat is also of importance. It may be significant that with the fat ration entirely cod-liver oil, no reproduction occurs and that the addition of 4 cc. of the oil to the diet results in a high mortality of the does. It is possible that vitamin E is as essential for normal birth as for effective placentation [Evans and Bishop, 1923, 1]. If the cod-liver oil has some inactivating action on the butter, similar to that suggested by Fridericia [1924] for certain oils, it might conceivably diminish the vitamin E content of the diet. Although this is a possible explanation of the results described in this paper, it is merely theoretical at the moment and the problem obviously awaits further elucidation. The evil effects consequent on the addition of cod-liver oil to the diet may be due to some upset of balance of the dietary constituents.

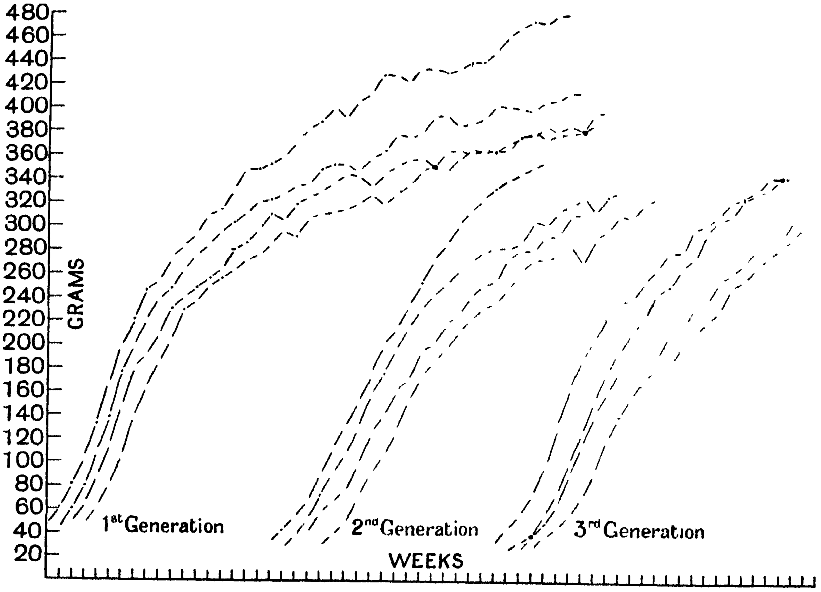

Fig. 3. Growth curves of some of the male rats fed on diet *A*.

The cod-liver oil was added in the hopes of preventing the sore eyes noticed in the suckling young, but no such improvement was obtained. More recently a similar condition has been found in suckling rats belonging to stock does fed on synthetic diets during lactation only. These mothers obviously cannot lack vitamin A as they have large reserves of body fat, so it is probable that the eye trouble is the "salt ophthalmia" described by McCollum, Simmonds and Becker [1922; 1925], although the conditions are not absolutely comparable, as these observers were feeding young rats on the diet, while in my experiments the eye trouble developed in the suckling young and was therefore caused indirectly by poor feeding of the mother rat. The soreness was observed as soon as the eyes opened (usually late on synthetic diets compared with stock animals). In some litters no bad eyes were seen, while in others two or three of the young showed the symptoms; in no case was the whole of the litter affected. In several experiments the eye trouble was noticed at the 17th day, and if the young ate for themselves on that day or the next, there was often complete recovery by weaning, *i.e.* the 21st day. This suggests that the diet was deficient for lactation though adequate for growth; it also emphasises the different needs of the rat during lactation and growth, because the young recovered when eating the diet for themselves.

It is possible that lactation throws an even greater strain on the mother than does gestation. In the rat, even if the litter be reduced to six, the combined weight of the young at weaning is sometimes equal to that of the mother, or in the case of a young animal, even greater, while the weight of a new-born litter is small compared with that of the mother, and yet gestation lasts for 23 and lactation 21 days. Thus a litter of six rats at 21 days of age would weigh from 240-300 g., while a new-born litter would rarely weigh over 70–75 g. It seems probable therefore that in a diet suitable for lactation, the carbohydrate should be decreased and other dietary factors increased as compared with the needs for growth.

Diet *A* is probably a good basis, which with some slight modifications, would make a suitable standard for nutrition work. This diet provides for good growth and reproduction. It is not satisfactory for lactation, but is fairly good for the gestation period as the young at birth were either of normal weight or not far below. The fact that the second and third generation animals grew rather less well than the first suggests that diet *A* is not complete, but as three generations were reared on the food it seems probable that all the necessary constituents are present, but that the balance of the various dietary factors needs adjustment. It is possible that a slight increase in mineral salts and perhaps vitamins as well (with a corresponding decrease of starch) is required during gestation, and a larger though similar increase for lactation. Only a few families were reared on diets *CE* and *CL*, but there is some evidence that this mixture of proteins gives better results during lactation, though for growth there seems to be no advantage in supplementing the caseinogen by other proteins. In fact diet *E* with only 16 % of caseinogen produced as good growth as diets *CE* and *CL* which contained 20 % of protein.

SUMMARY.

1. Three generations of rats have been reared on a synthetic diet of butter, caseinogen, potato starch, salt mixture, marmite and distilled water.

2. The use of cod-liver oil instead of butter in this synthetic diet produces less good growth and causes sterility.

3. Synthetic diets are described which produce good growth in young rats, but cause inability of the young to be born and consequent death of the doe.

4. Further evidence is given that the dietary requirements of the rat vary at different phases of its existence.

The expenses of this research were defrayed by a grant from the Medical Research Council. I am indebted to Miss W. M. Clifford for making the *post mortem* examinations in connection with this research.

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CXLIV. THE PHOTOSYNTHESIS OF UREA FROM AMMONIUM CARBONATE.

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THE formation of urea from ammonium carbonate in aqueous solutions at temperatures below 50° is a reaction of considerable biochemical interest in connection with the obscure mechanism of the formation of urea in the animal body.

Under these conditions urea has been obtained from ammonium carbonate or bicarbonate by a variety of apparently unrelated methods, which may be summarised as follows.

1. Electrolysis, using an alternating current. Drechsel [1880].
2. Electrolysis, using a direct current. Millot [1885]; Fichter, Steiger and Stanisch [1918].
3. Zymolysis, using concentrated preparations of urease. Kay [1923]; Mack and Villars [1923].
4. Incubation with various charcoals. Fichter and Kern [1925].
5. Incubation in a sealed tube for 12 months. Werner [Fearon, 1926].

As a result of the present investigation it has been found possible to detect urea in concentrated solutions of ammonium carbonate after ultraviolet irradiation for periods of 2–10 hours. This is in agreement with the conclusions of Bailey, who detected traces of urea after the exposure of gaseous mixtures of ammonia and carbon dioxide to radium emanation [1925] and to ultra-violet irradiation [1927].

EXPERIMENTAL.

Reagents and apparatus.

1. The ammonium carbonate employed was the purest form supplied by Kahlbaum. It was not analysed for carbamate or bicarbonate.
2. The xanthhydrol was obtained from Poulenc. It was used in 10 % concentration in methyl alcohol.
3. The urease used was the Dunning preparation.
4. The source of irradiation was a Hanovia "Alpine Sun" A.C. mercury vapour lamp. To compensate for any decrease in the efficiency of the lamp the quartz arc tube was replaced by a new one early in the investigation.

Method.

The solutions were irradiated in a flat porcelain dish, 21 cm. in diameter, on account of the observation made by Fichter and Kern [1925] that the prolonged action of warm, concentrated ammoniacal solutions is liable to extract silicic matter from glass-ware, and thus interfere with the specificity of the xanthhydrol test. The distances of the solutions from the lamp were such that the temperature of the liquid did not rise above 45°. In most of the experiments the temperature range was 30°–40°.

After irradiation, the solutions were concentrated to about 5 cc. on a water-bath, and either tested directly by means of urease, or treated with four volumes of glacial acetic acid and filtered free from dust particles. 2 cc. of the 10 % xanthhydrol solution were then added drop by drop and the mixture having been shaken was allowed to stand for 12 hours. Where a precipitate was obtained its identity as dixanthylurea was established by the following tests:

(1) the characteristic sheen of the precipitate, and its microcrystalline nature;

(2) the rosy colour of the warm residue when dried on the filter paper;

(3) the melting-point (260°) as determined in the vapour of diphenyl ether.

Results.

It was found easy to demonstrate the formation of urea in 10 % and 20 % solutions of ammonium carbonate after irradiation of 200 cc. for periods of 2 to 6 hours. The urease test was positive, and the precipitates of dixanthylurea were definite although very small, being of the order of 1–5 mg. per 200 cc.

The control experiments of non-irradiated ammonium carbonate solutions gave no trace of urea after concentration.

250 cc. of a 10 % solution of ammonium carbonate which had been kept in a glass flask exposed to daylight for one year was found to be free from urea.

The effect of various accessory reagents on the reaction was then examined. Glucose, hydroxylamine and formaldehyde were selected as reducing agents; methylene blue, ammonium nitrate and hydrogen peroxide as oxidising agents; copper, iron, and uranium as possible metallic catalysts; methyl orange, malachite green, and eosin as possible photo-catalysts.

The principal results are summarised in Table I.

Of all the accessory reagents examined, malachite green had the most definite effect in increasing the yield of dixanthylurea. During the irradiation the solutions effervesced in a manner not observed with any of the other dye-stuffs examined. Accordingly, these experiments were repeated with controls, and the weight of dixanthylurea determined. After irradiation, the unchanged dye-stuff was removed by acidifying the concentrated solution with acetic acid and extracting with chloroform.

Exps. 3 and 4 are controls, and indicate that the urea did not originate from the action of the ammonium carbonate on the dye-stuff in the absence of the irradiation, or from the irradiation of the dye-stuff in the absence of the ammonium carbonate.

Table I. *Irradiation of ammonium carbonate.*

Time, 2 hours. Temperature, 10°—40°. Distance from lamp, d cm.

Expt.	$(NH_4)_2CO_3$ g.	H_2O cc.	d cm.	Urea	Accessory reagent
1	10	100	25	+	None
2	20	200	15	+	None
3	36	120	15	+ +	None
4	8	100	25	+	Glucose, 2 g.
5	20	100	15	–	$NH_2OH.HCl$, 1 g.
6	40	200	10	–	NH_4NO_3, 1 g.
7	40	200	20	+ +	(Copper stirrer)
8	40	200	10	+	Uranium acetate, 0·2 g.
9	20	100	10	+	Eosin, 0·001 g.
10	40	200	10	+ + +	Malachite green, 0·04 g.
11	40	200	10	+	Methyl orange, 0·03 g.
12	10	100	25	–	Methylene blue, 0·01 g.

Table II. *Photocatalytic action of malachite green.*

Time, 10 hours. Temperature, 10°–40°. Distance from lamp, d cm. Vol. of solution, 200 cc.

Expt.	$(NH_4)_2CO_3$ g.	Dixanthylurea g.	Malachite green g.	d
1	40	0·061	0·02	10
2	40	0·046	0·01	8
3	40	0·000	0·01	infinity
4	0	0·000	0·01	10

DISCUSSION.

In the absence of more adequate data it appears premature to discuss the possible mechanisms concerned in these low temperature syntheses of urea. A general conclusion is that urea is a stable end-product which may be formed in various ways in systems containing ammonia and carbonic acid. Two such ways are (1) alternate reduction and oxidation of carbamic acid, amounting to indirect dehydration; and (2) direct dehydration of carbamic acid. The former process offers an explanation of the production of urea during the electrolysis of solutions of ammonium carbonate, and also, it is believed, is concerned in the photosynthesis of urea.

The latter process, direct dehydration of carbamic acid, is probably responsible for the synthesis of urea by the action of urease, active charcoal, or incubation in a sealed tube.

No attempt was made in the present investigation to determine the range of the ultra-violet spectrum concerned in the synthesis of urea. It was observed, however, that the efficiency of the quartz lamps decreased notably during a lengthy series of experiments, and eventually the photosynthetic properties became negligible[1].

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[1] Note added Sept. 18th, 1927. By means of the ultra-violet spectroscope it was found that the effective radiations were between 250 $\mu\mu$ and 200 $\mu\mu$. When a glass screen was interposed, or when the lamp had aged sufficiently, the radiations did not extend into the ultra-violet beyond 250 $\mu\mu$. Under these conditions no urea could be obtained photosynthetically.

SUMMARY.

1. Urea has been produced by the ultra-violet irradiation of solutions of ammonium carbonate at temperatures not exceeding 45°.

2. The yield of urea was increased by the presence of malachite green, but not by that of methylene blue, methyl orange, or eosin.

3. The effective radiations for the synthesis of urea lie between 250 $\mu\mu$ and 200 $\mu\mu$.

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CXLV. THE EFFECT OF CERTAIN SUGAR DERIVATIVES ON INSULINISED MICE.

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In their researches on the efficiency of various sugars and their derivatives in relieving the symptoms caused by insulin in mice, Herring, Irvine and Macleod [1924] made no use of nitrogenous derivatives. Consequently, early in the spring of 1925, a series of experiments was carried out to test the effect of such compounds, but the results then obtained were not reported owing to their negative character. However, as, recently, other workers have published researches covering the same field, the following results are now communicated.

As a large number of the better known nitrogenous sugar derivatives contain their nitrogen in a group which is foreign and toxic to the animal organism, the compounds investigated were limited to glucose derivatives, in which the nitrogen was present either as an NH_2-group or in the form of urea.

In carrying out the experiments the technique adopted was identical with that described by Herring and co-workers, except that control experiments with normal mice were carried out with each of the compounds tested. These were: (1) glucosimine, (2) glucose ureide, (3) glucosamine hydrochloride, and (4) chitose. A 15–20 % solution of each was employed, 5–10 minims being used for an injection.

(1) d-*Glucosimine.*

The glucosimine used for the first experiments was prepared by Lobry de Bruyn's method [1895], and, as the melting point of the specimen agreed with that quoted (127–128° with decomposition), the material was taken as sufficiently pure for use. Its injection into mice rendered comatose by insulin brought about slight and temporary relief, but this was subsequently shown to be due in all probability to a slight contamination with glucose since a specimen of glucosimine (M.P. 129°), prepared by Ling and Nanji's method [1922] and then recrystallised from methyl alcohol, not only was incapable of producing any relief, but seemed to exert a toxic action.

The specific rotation of the first specimen of glucosimine was found to be $+ 23{\cdot}61°$ ($c = 2{\cdot}287$), that of the purified glucosimine was $+ 19{\cdot}87°$ ($c = 1{\cdot}928$), which agrees with that quoted by Lobry de Bruyn, namely $+ 19{\cdot}5°$. Consequently, a specimen of glucosimine, although possessing the required melting point, cannot be taken as reliable unless it is known that the $[\alpha]_D$ is less than $+ 20°$.

(2) d-*Glucose ureide.*

The glucose ureide employed was perfectly pure (M.P. 207°, $[\alpha]_D = -23\cdot44°$) [Hynd, 1926]. This compound had no effect on either normal or insulinised mice.

(3) d-*Glucosamine hydrochloride.*

No symptoms were detected on injecting the pure salt into normal mice. Four mice suffering from insulin convulsions were in no way benefited by injection of this compound, but recovered slowly after the administration of glucose. Moschini [1924] has recorded similar findings. As this worker found that a previous injection of this compound diminished and delayed the effect of insulin on rabbits, he concluded that glucosamine is transformed slowly into glucose *in vivo*. This appears to be opposed to the generally accepted view as to the behaviour of glucosamine in the animal organism, a summary of which is given by Levene [1925]. An alternative explanation is that glucosamine hydrochloride, just like ammonium chloride, would tend to produce an acidosis, which would render the rabbit somewhat more resistant to the action of insulin [Abderhalden and Wertheimer, 1924; Hetényi, 1926].

(4) *Chitose.*

Chitose syrup was tested on one normal and on six insulinised mice. It was quite inactive.

DISCUSSION.

Despite the negative character of the above results, they are not without interest when the close relationship of all four compounds to glucose is considered. In both glucosimine and glucosamine, the glucose molecule is altered only by the substitution of an NH_2-group for an OH-group. Yet this is sufficient to render the molecule inactive.

As all the evidence points to the reducing group being essential, its substitution in the case of glucose ureide and glucosimine made it unlikely that these derivatives would have any efficiency. However, these two compounds differ from those hitherto examined with the reducing group substituted, in that they are relatively unstable in alkaline solutions, and might, therefore, have been expected to produce a positive result under the conditions obtaining in the animal body.

The third nitrogenous compound employed, namely glucosamine, conforms to the general formula arrived at by Irvine for a compound which should alleviate the symptoms produced by insulin, as the only alteration is at the second carbon atom. The groups at this position have so far been regarded as immaterial, but it is evident that while it is permissible to have inversion of the OH-group at the second carbon atom, this OH-group cannot be substituted without destroying the efficiency of the compound.

The fourth compound examined, namely chitose, is nitrogen-free, but again closely related as it is obtained by the deamination of glucosamine, and may be regarded as 2.5-anhydro-glucose (or -mannose). Chitose is thus the third example of an anhydro-sugar which has been tested as to its efficiency for relieving insulin symptoms. Winter [1926] carried out experiments with α-glucosan, while the action of β-glucosan was investigated at an earlier date by Herring, Irvine and Macleod [1924]. Negative results were obtained in both these cases, presumably because the reducing group was absent. The inactivity of chitose cannot be explained in this way, as the reducing group is free. Further, as the correct configuration is preserved, the lack of efficiency must be referred to the ring structure which is present in the compound.

It is clear that glucose is by far the most efficient sugar for counteracting the action of an excessive amount of insulin, and any alteration of its molecule, except changes at the second carbon atom to give either mannose or glucal, which is converted readily into mannose [Winter, 1926], lowers the efficiency. Though changes at the second carbon atom are involved in glucosamine and chitose, the non-activity of these two compounds is not surprising. Accordingly it may be concluded that the introduction of nitrogen (*e.g.* glucosimine, glucosamine and glucose ureide) or of a second ring structure (*e.g.* chitose, α-glucosan and β-glucosan) into the glucose molecule completely destroys the power of relieving insulin hypoglycæmia.

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CXLVI. THE ANTAGONISM OF GLUCOSONE AND CYANIDES *IN VIVO*.

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Recently many references have appeared in the literature to the antagonistic action towards cyanides which results from the previous administration of glucose either alone, or in conjunction with insulin. As, in the course of experiments on the physiological action of glucosone [Hynd, 1927], it had been noticed that an antagonism also appeared to exist between glucosone and potassium cyanide, the question was investigated more fully in the hope of correlating the results referred to above.

Owing to the small amount of glucosone available, the majority of the experiments involving the use of this compound were carried out on mice, but a certain number were performed on young albino rats and also on rabbits. In the experiments described 67 mice, 14 rats and 14 rabbits have been employed.

I. The subcutaneous injection of potassium cyanide into mice.

In the first experiments potassium cyanide was administered subcutaneously. The mice used were found to be much more resistant to this poison than was expected, and consequently a number of preliminary trials had to be made to determine the necessary dosage for the object in view. From these it appeared that 4 minims of a 0·5 % solution of potassium cyanide was a suitable dose for a mouse weighing 20 g. Such a dose of cyanide rarely renders a mouse moribund, but nevertheless it produces, in a short time, very definite symptoms, usually the first to be noted being a slight retraction of the head and "biting at the air." Within five minutes after the injection leg-weakness is noticeable, and this soon becomes more marked so that in the course of another five minutes the animal is unable to walk—any attempt to do so being followed by severe tremors. Handling of the mouse at this stage also induces tremors, after which the animal sometimes rolls over and lies on its back. Return to the prone position may not occur for some time, but it usually takes place suddenly, and is sometimes accompanied by a convulsion. Thereafter recovery takes place slowly and the mouse appears fairly normal in about two hours after the injection.

Three mice of approximately equal weight were selected for each experiment—the first being injected with cyanide, the second with a suitable amount of glucosone, and the third receiving injections of the same amounts of both cyanide and glucosone.

An 11·35 % solution of glucosone was used and 0·3 of a minim per g. body-weight was the amount injected, as such a dose was known to be nonlethal but capable of producing severe symptoms. This always was realised in the animals receiving glucosone alone—marked sprawling of the legs being evident in 5 to 10 minutes after injection and more severe symptoms in the course of 15 to 20 minutes.

Animals receiving injections of both glucosone and cyanide were from 20 to 30 minutes before exhibiting any symptoms. Usually a slight glucosone effect, such as sprawling of the legs, was observed 20 minutes after the injection instead of in 5 minutes in the control animals, and no cyanide effect was detected till 30 minutes had elapsed. The symptoms displayed were of a mild character and of a mixed type, at one moment suggesting the cyanide, at another the glucosone action, but return to the normal required a somewhat longer period than was the case with a mouse receiving potassium cyanide alone.

This experiment has been repeated on three separate sets of animals with uniform results.

II. THE SUBCUTANEOUS INJECTION OF ACETONITRILE.

A series of experiments was then carried out in which the alkali cyanide was replaced by acetonitrile. As the result of preliminary experiments it was found that a dose of 0·2 minim of a 5 % solution of acetonitrile per g. of body-weight produced in a mouse a fairly characteristic train of symptoms. These included tremors, lowering of body temperature, sprawling limbs and convulsions.

As before, three mice of approximately the same weight were used in each experiment—the first being injected with acetonitrile, the second with acetonitrile and glucose, and the third with acetonitrile and glucosone. All the animals used in this set of experiments exhibited in about 20 minutes marked sprawling of both fore and hind limbs, while at a later stage those not receiving glucosone, as a rule, rolled over on their backs and lay clasping the fore limbs together. The "glucosoned" mice showed no sign of tremors, which was a striking feature in the other animals. Moreover, though on some occasions, half an hour after being injected, the mice which had received glucosone appeared most affected, after 3 to 4 hours they were quite normal, whereas the others still exhibited effects of the nitrile (including diarrhoea) and, in some cases, the mice injected with acetonitrile alone were found dead the following morning.

III. THE EFFECT OF AN ATMOSPHERE CONTAINING HYDROGEN CYANIDE.

The effect of the inhalation of hydrogen cyanide was then studied on mice. For this purpose a bell-jar of 7 l. capacity, closed with a ground glass plate and fitted with a hydrogen cyanide generator, was used—the latter being so arranged that the hydrocyanic acid was liberated only after the experimental animals had been introduced into the bell-jar. By trial it was found that the gas evolved from the interaction of 3 cc. of 4 % potassium cyanide and 2 cc. of 40 % sulphuric acid, furnished a suitable concentration of hydrogen cyanide. Although the exact concentration of the hydrogen cyanide was unknown, this was immaterial, as three animals were used in each experiment—the first injected with a non-lethal dose of glucosone, the second with an equivalent amount of glucose, and the third not injected. Animals of equal weight were chosen as far as possible, and where this could not be managed, the lightest received glucosone and the heaviest was used as the normal animal. Eight experiments of this type have been carried out and invariably it has been found that glucose affords no protection against hydrogen cyanide. On the other hand, a subcutaneous injection of glucosone enables a mouse to resist the toxic action of hydrogen cyanide. These facts are clearly demonstrated in the following summary of a more or less typical experiment.

Effect of an atmosphere containing hydrogen cyanide.

Time a.m.	Black mouse 20·0 g.	White mouse 19·4 g.	Grey and white mouse 19·6 g.
10.9	No injection	3 minims 18·13 % glucosone	3 minims 20 % glucose
10.15	HCN atmosphere	HCN atmosphere	HCN atmosphere
10.16	Running about sniffing	Sitting with head down	Appears normal
10.19	Respiration very rapid: beginning of cyanide effect	Slight sprawling of legs: due to glucosone	Appears drowsy
10.24	Leaping about	Slight sprawling of legs: due to glucosone	Walking about
10.28	Sitting gasping: still able to cling	Still sprawling but can walk about when disturbed	Beginning to show effect of cyanide
10.30	Lying on side: gasping	Slightly improved but eyes protruding	Violent convulsion: then rolls over on to its side
10.32	Symptoms very severe: slight convulsion	Slightly improved but eyes protruding	Respirations very slow
10.34	Rolls over: several severe convulsions	Slightly improved but eyes protruding	Eyes protruding
10.52	Lying moribund	Beginning to show slight cyanide effect	Dead
	Removed to ordinary air.	*Removed to ordinary air.*	
10.56	Severe convulsions	Sitting in normal position but unwilling to move	
11.0	Slightly improved	Definitely improved	
11.5	Still blind: severe tremors when handled	No tremors, but signs of diarrhoea	
11.10	Several convulsions	Fairly normal	
11.30	Improving generally: eyes normal	Fairly normal	
p.m. 2.0	Practically normal	Drowsy: fur ruffled	

Certain of the experiments carried out have been even more striking than the above, as, for example, that in which a normal mouse of 26 g. weight was rendered moribund in 10 minutes by the hydrogen cyanide vapour, while a 20 g. mouse, which had been injected with glucosone, showed only slight symptoms after 2½ hours' inhalation, at which time the normal mouse died.

Very similar results have been obtained in three experiments with young albino rats of an average weight of 35 g. and from the same litter, and also in one experiment when the average weight was 75 g. All the rats used in these experiments survived, but the striking feature was the rapidity with which those injected with glucosone became normal on removal from the atmosphere of hydrogen cyanide. The recoveries of both the "normal" rat and that injected with glucose were in comparison slow, and moreover were accompanied by a number of severe convulsions.

A number of experiments were also carried out to determine whether insulin with or without glucose furnished any protection for mice exposed to the vapour of hydrogen cyanide. Doses of insulin varying from 0·8 to 1·6 units per 100 g. body-weight were employed, but no definite protective action could be demonstrated. In fact all the insulinised mice, which were exposed to gaseous hydrocyanic acid, died, while normal mice, though displaying severe cyanide symptoms as the result of the same exposure, recovered on removal to ordinary air. In the majority of the experiments it appeared that exposure to the atmosphere of hydrogen cyanide hastened the development of the insulin symptoms, but this point could not be established by control experiments.

IV. Experiments involving the intravenous injection of potassium cyanide into rabbits.

(i) *The antagonistic action of insulin plus glucose.*

(*a*) A rabbit of 2·7 kg. was injected intravenously with 30 units of insulin and 4 cc. of a 40 % solution of glucose. Eight minutes later, 5 minims of a 4 % solution of potassium cyanide were injected into the same ear vein. Immediately the animal showed difficulty in breathing and, as soon as released, it rolled over on its side. The head was retracted and respirations were very slow, but in 2 to 3 minutes they improved slightly. This was followed a minute later by a severe convulsion, and two minutes afterwards the animal was able to sit up, although the hind limbs were still extended. Ten minutes later the rabbit looked quite normal and no further symptoms were noticed during the course of the day.

Two days later, using the same rabbit, the experiment was repeated except that no insulin was administered. The animal behaved exactly as on the previous occasion—severe symptoms immediately after the cyanide injection and return to the normal in about 10 minutes.

(*b*) The above experiment was repeated with a larger dose of cyanide (8 minims of 4 % KCN) using two rabbits weighing 2720 g. and 2940 g.

respectively—the former, a male, receiving 30 units of insulin and the latter, a female, no insulin. They behaved in exactly the same manner, except that after returning apparently to the normal condition the insulinised male rabbit showed slight insulin symptoms but recovered without any treatment.

(*c*) Having found that the intravenous injection of 5 minims 10 % potassium cyanide into a rabbit (♂) weighing 3 kg. caused death almost instantaneously, another rabbit (♀) of the same weight was injected intravenously with 4 cc. of 40 % glucose and 30 units of insulin, and five minutes later 5 minims 10 % potassium cyanide were introduced into the marginal vein of the other ear. Severe symptoms ensued immediately, but 20 minutes later the animal was fairly normal, its behaviour having been very similar to that of the first rabbit described above in experiment (*a*).

Five days later this experiment was repeated on the same animal with identical results. After another period of five days the same rabbit was used to study the effect of the same amount of cyanide after glucose but without insulin. Complete though somewhat slow recovery took place, the rabbit being able to sit up in the normal position 45 minutes after the injection. About a fortnight later, on injecting the same amount of cyanide alone, this rabbit collapsed at once and died in one and a half minutes.

(ii) *The antagonistic action of glucosone.*

(*a*) A young rabbit weighing 900 g. was injected intravenously with 1·5 cc. of 18·13 % glucosone[1], and then immediately afterwards with 3 minims of 5 % potassium cyanide. Severe struggling took place for about half a minute, thereafter the animal lay on its side quite collapsed. After one minute the corneal reflex returned. No twitching or tremors were noticed, but the rabbit lay quite limp for about ten minutes, at which time it was able to hold up its head, although the limbs remained limp till about five minutes later. Twenty minutes after receiving the injection, the animal appeared drowsy but otherwise normal.

A week later the effect of the same dose of cyanide was studied on the same rabbit. As before severe struggling took place, followed by collapse, the animal lying on its side helpless, with head retracted and eyes fixed. Respirations, which at first were slow, gradually improved in the course of 2 to 3 minutes, during which time a number of inspiratory cries were uttered. Five minutes elapsed before the reflexes returned: in ten minutes, after marked twitching especially of the fore limbs, an attempt was made to sit up, but after doing so the rabbit gradually fell over again on its side. Two minutes later, however, it succeeded in resuming the normal position but was not quite recovered in half an hour, being disinclined to move, sitting with ruffled fur and having a general scared appearance.

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[1] So far the physiological action of glucosone has been reported only on mice [Hynd, 1927]. Its action on other species has been investigated and an account of this is at present in preparation.

(*b*) Two young rabbits of the same litter and of approximately the same weight were used for the next experiment. One (530 g.), on injection with 1·6 minims of 5 % sodium cyanide, displayed the usual symptoms and died in 1½ minutes. The other (550 g.) was given intravenously 1 cc. of 18·13 % glucosone and five minutes later 1·6 minims of 5 % sodium cyanide. Less severe struggling ensued and the animal died within a minute of receiving the cyanide.

(*c*) In the following experiment the dosage of cyanide was reduced by half, and two rabbits of the same weight (620 g.) and from the same litter were employed.

20 minims tap-water mixed with 1·7 minims 2·5 % sodium cyanide were injected into the left ear vein of one animal. After half a minute's violent struggling the rabbit lay collapsed on its side. Improvement in respiration was noted in three minutes, but reflexes did not return for 11 minutes. Efforts to hold the head erect were not made until 18 minutes had elapsed, and at this time both fore and hind limbs were still sprawling. Return to a fairly normal condition required about 50 minutes.

20 minims 18·13 % glucosone mixed with 1·7 minims 2·5 % sodium cyanide were similarly injected into the other rabbit. Struggling was not nearly so marked and the animal never collapsed. Reflexes were never absent, the head was kept always erect, but the limbs were extended for a few minutes. Three minutes after the injection the rabbit seemed fairly normal but looked frightened.

Two days later the treatment of the animals was reversed. On giving the same dose of glucosone and cyanide to the first rabbit slight struggling occurred during the injection but otherwise no effect was noticed. The second rabbit, on the other hand, struggled so violently during the injection of cyanide alone that only half the amount was introduced and the animal died within a minute.

Three days later, similar behaviour occurred with the surviving rabbit when it was injected with 1·7 minims of 2·5 % sodium cyanide previously diluted with 18 minims of tap-water. After very violent struggling during the injection, the animal collapsed and died almost instantaneously.

DISCUSSION.

The view expressed by Horvath [1926] that the therapeutic value of glucose consists in part in an antitoxic rôle toward the CN-group, is not borne out by the results of the experiments now described. In no case has there been observed any definite protective action of glucose against alkali cyanide, gaseous hydrocyanic acid, or acetonitrile. This is also contrary to the findings of Violle [1926], who has stated that, while a concentration of 2 g. hydrogen cyanide per cubic metre of air will kill a rabbit in a few minutes, animals previously fed or injected with glucose can breathe this toxic atmosphere for more than an hour without ill effects. As the concentration of hydrogen cyanide used in his experiments was of the same order as that employed by the present author, there is the possibility that the divergence in the results might be explained by the fact that the experiments were conducted on a different species of animal, for, while Violle used rabbits, my comparable experiments were confined to mice and rats. This explanation, however, seems unlikely as already Voegtlin, Johnson and Dyer [1926] and also Heymans and Soenen [1927], who used rabbits and guinea-pigs, have described experiments, the results of which point to glucose being incapable of counteracting the toxic effects of cyanogen compounds.

On the other hand, the results of the present investigation point to the fact that glucosone and potassium cyanide are antagonistic in their actions in the animal organism, for the symptoms accruing from either compound are delayed in their development and diminished in their intensity when simultaneous injections of these substances are given subcutaneously.

Evidence that glucosone counteracts the CN-group is also furnished by the experiments with acetonitrile. These were performed in the hope that this somewhat less toxic compound would yield even more definite results than those obtained with the alkali cyanide. This was not realised, however, and, in fact, the results were somewhat less definite.

Although controls were always carried out against experiments involving the subcutaneous injection of cyanides, it was difficult, especially when dealing with small animals such as mice, to be certain that the proper amount was injected, and even though this operation had been accomplished successfully, there was no guarantee that the rate of absorption would be comparable in the experimental and the control animal. However, any objection on this account is negatived owing to the number of experiments carried out and the uniformity of the results obtained.

Further evidence of a convincing character, as to the antagonistic action of glucosone and cyanide, is furnished by the results of the experiments in which the experimental animals were exposed to an atmosphere containing hydrogen cyanide. Though very definite results were obtained in these inhalation experiments with both mice and rats, the dose of glucosone required to afford protection is a fairly large one, being only slightly less than what would be a lethal dose under normal atmospheric conditions. This may explain why insulin with or without glucose, or even glucose itself, has been found to be useless for small animals, as sufficient glucosone would not be produced quickly enough to counteract the large amount of cyanide. The author's suggestion [Hynd, 1927] that insulin converts inactive glucose into utilisable glucosone, may provide the key for the explanation of the results reported by Szolnoki [1926] and by Rosenberg [1926] regarding the antidotal action of insulin in hydrocyanic acid poisoning.

Reference to the experimental part dealing with the intravenous injection of potassium cyanide into rabbits will show that attempts to repeat the experiments described by Rosenberg [1926] were not altogether successful, as in the third experiment the rabbit recovered from the cyanide injection when protected by glucose without insulin. The symptoms which ensue after an intravenous injection into a rabbit of an amount of cyanide approaching the lethal dose are so severe that recovery of the animal appears to be always rather problematic, no matter what substance has been administered for its protection. So large a number of factors are involved, as for example, the rate at which the cyanide is injected and its subsequent distribution, that one must hesitate in drawing too definite conclusions.

Nevertheless, taking the results of the present experiments as a whole, it seems established that glucosone exerts an antagonistic action towards the CN-group. In this respect, however, glucosone is not so efficient as, for example, cystine, with which it was compared and which, as Voegtlin and his co-workers have shown, is able to protect rats against lethal doses of cyanide. Consequently, glucosone in no sense can be recommended as an antidote in hydrocyanic acid poisoning, and evidently the use of glucose or of glucose plus insulin would be of even less avail for such a purpose.

It is now generally accepted that the normal mechanism of detoxicating cyanides in the animal body involves interaction with sulphur compounds. Although this may be true under normal conditions, it seems possible that, when excessive amounts of cyanides have to be dealt with, the supply of such thio-compounds may be inadequate and carbohydrate may then be called upon, just as glycuronates appear in the urine when there is produced an excess of compounds which normally are eliminated as ethereal sulphates. As in glucosone, an oxidation product of glucose, the body would have at its command an efficient detoxicating agent, it seems possible, in view of the results already obtained [Hynd, 1927], that glucosone is a compound of real physiological importance.

SUMMARY.

1. In the case of mice and rats, a subcutaneous injection of glucose, or of glucose plus insulin, affords no protection against the toxic action following (*a*) the subsequent subcutaneous injection of either alkali or methyl cyanides, or (*b*) the inhalation of gaseous hydrogen cyanide.

2. Under the same experimental conditions, glucosone exerts a definite antagonistic action towards cyanides, but is less efficient than cystine.

3. The previous, or simultaneous, intravenous administration of glucosone, or of glucose plus insulin, appears to be protective against the intravenous injection of alkali cyanide into rabbits, but the results obtained are somewhat variable.

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CXLVII. THE SYNTHESIS OF ANTINEURITIC VITAMIN BY YEAST.

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(*Received August 19th, 1927.*)

RECENTLY Hawking [1927] has shown that yeast can synthesise the factor which cures the symptoms of head retraction in pigeons fed upon polished rice. These experiments were not quite conclusive on two accounts, viz.: (1) the amount of "bios extract" used in growing the yeast contained so much of the antineuritic factor that the amount of the latter which was synthesised appeared to be small; and (2) there was some contamination of the yeast by bacteria. The following experiment was performed under conditions which were more satisfactory in these respects.

EXPERIMENTAL.

The cane sugar, salts and "bios extract" were precisely the same as Hawking employed, and the experiment may be regarded as a continuation of his work. A basal medium was used, of which the composition was:

KH_2PO_4	5·0 g.	$CaCl_2$	0·25 g.
NH_4Cl	2·5 g.	Cane sugar	50·0 g.
$MgSO_4$	0·35 g.	Glass-distilled water to	1000 cc.

The yeast was the same strain of *Saccharomyces cerevisiae*, and the stock cultures were grown at 25° in 5 cc. portions of basal medium containing 0·05 cc. of "bios extract." A small loopful of stock culture (21 days old) was transferred to a fresh tube of this medium and grown at 25° for one day. The supernatant fluid was pipetted off and replaced by 5 cc. of basal medium, the resulting suspension being used for inoculation of the flask in the following experiment.

Experiment. 0·5 cc. of "bios extract" was added to 1500 cc. of basal medium in a 2-litre flask, the neck of which had been drawn out to a diameter of 15 mm. in order to minimise the risks of accidental infection. After sterilisation (by steam-heat on three successive days) the flask was inoculated with approximately 3000 yeast cells and maintained at 25° for 10 days with occasional shaking. Samples were then removed to agar slopes, and the culture was at the same time examined in wet and dry films. The latter showed that there was no bacterial contamination, and the growth on the agar slopes consisted in all cases of pure yeast. The greater part of the fluid was syphoned off, and the remainder separated on the centrifuge. The resulting yeast weighed 5·7 g. (wet). This yeast was immediately extracted by boiling for one minute with 7 cc. of 1 % acetic acid, and the extract separated from the cell-residue by centrifuging. The fluid was decanted, made up to a volume of 10 cc. with water, and divided into two equal parts.

Two pigeons were used, each receiving 5 cc. of the extract. In both the symptoms of head retraction disappeared, to reappear in one case after 2·75, and the other after 3·25 days. The yeast therefore contained at least 6 day doses.

Hawking's experiments showed that the "bios extract" did not contain more than 0·7 day doses in 0·5 cc. The cells used for inoculation represented a thousandth part of the suspension from which they were obtained, and therefore did not contain more than 0·00007 day doses. Thus, before the development of the yeast, the flask did not contain more than 0·70007 day doses. Of the 6 day doses found in the yeast, 5·3 day doses were presumably synthesised.

SUMMARY.

An experiment is recorded in which 5·7 g. of yeast were produced in a medium which did not contain more than 0·7 day doses of antineuritic vitamin. Bacterial contamination was avoided, and the yeast contained at least 6 day doses. Considerable synthesis can therefore occur.

I wish to thank Prof. Peters for his interest and advice, and for his kindness in testing the extract on the pigeons.

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CXLVIII. KIDNEY PHOSPHATASE.

II. THE ENZYME IN DISEASE.

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(*Received August 24th, 1927.*)

In an investigation recently described by one of us in this *Journal* [Kay, 1926] data were quoted which strongly suggested a correlation between the phosphatase content of the kidney and the functional activity of this organ.

Further support for this suggestion has now been obtained from a study of the phosphatase content of (*a*) human kidneys, obtained *post mortem* from individuals dying from various causes, including kidney disease, (*b*) kidneys of rabbits with acute experimental nephritis.

Method.

The method used for determining the phosphatase content of a given kidney is that described on pp. 795 and 797 of the communication just mentioned. The activity of the phosphatase of rabbit's, rat's and pig's kidney has been previously shown to have a fairly broad optimum between p_H 8·8 and 9·2. This is true also for human kidney phosphatase. The optimum reaction becomes somewhat less alkaline as the period over which the activity of the enzyme is determined increases. In the great majority of the determinations which are given in the present paper, the activity of each kidney extract has been ascertained at a series of p_H values from 8·25 to 10·0. For two hours' activity the optimum is invariably between p_H 8·8 and 9·2. The unit of kidney phosphatase we define as the amount of enzyme which, at 38°, and in Sørensen's glycine-NaOH buffer solution at p_H 8·9, is required to liberate 1 mg. phosphorus as inorganic phosphate from excess of sodium β-glycerophosphate solution in 2 hours. The phosphatase activity of a tissue we express in units per g. (wet weight). Provided care is exercised in the sampling of the tissue (which should be obtained with as little delay as possible *post mortem*), analyses by different observers on the same kidney give reasonably concordant results (Table I).

Table I. *Kidney phosphatase in units per g. tissue (wet weight).*

	Observer			
No. of expt.	R. T. B.	H. D. K.	R. H. R.	Remarks
1	2·6	2·7	—	Normal
31	0·5	0·4	—	Nephritic
A 241	—	4·7	4·9	Normal
A 192	2·6	2·8	—	Cardiac failure "back pressure" kidney

If a portion of a rabbit's kidney is kept for 24 hours at room temperature before making the determination, there is usually a slight fall (about 10 %)

in the phosphatase activity. The human kidneys used in this series were obtained at the routine *post mortem* examination, usually within 24 hours of death, during which time the cadavers were kept in a cold chamber. With human kidney a further period of 24 hours in the cold box in addition to the usual time between death and *post mortem* examination has no appreciable effect on phosphatase activity. It is therefore unlikely that variations in the phosphatase content of the kidneys, due to *post mortem* changes, exceed 10 % in any of our determinations.

A. Human Kidneys.

Results of determinations carried out on portions of kidney obtained *post mortem* from cases of disease and of accidental death are shown in Tables II, III, IV, and V.

Table II. *Cases without definite clinical or* post mortem *evidence of renal disease or abnormality other than slight parenchymatous degeneration.*

No. of expt.	Age of individual	Cause of death	Report on kidneys *p. m.*	Phosphatase units per g.
A 67	6½ months foetus	Foetus quite healthy. Premature delivery	Normal	1·0
A 80	2 full term foetuses	Delivery	,,	1·8 2·4
A 180	5 hours	—	,,	0·8
17	2 days	Haemorrhage	Anaemia*	1·9
1	6 years	Fractured skull	Normal	2·7
A 241	6 ,,	Injuries	,,	4·7
5	10 ,,	Abscess of brain	,,	4·6
6	Adult	Heart disease	,,	5·4
10	52 years	Injuries	One kidney lacerated, other normal (latter used)	6·2
9	60 ,,	Cerebral tumour	Parenchy. degeneration	3·3
A 11	10 ,,	Injuries	To naked eye, normal	5·8
24	3 months	Diarrhoea and vomiting	Parenchy. degeneration	5·8
25	18 years	Injuries	Anaemia*	6·2
32	35 ,,	Haemorrhage from gastric ulcer	,, *	5·1
A 202	46 ,,	Cerebral tumour	Normal	4·8

* Due to pre-mortal haemorrhage.

Table III. *Cases in which death was due to nephritis.*

No. of expt.	Age of individual	Cause of death	Report on kidneys	Phosphatase activity
2	50	Uraemia	Slightly narrowed cortex	1·6
3	18	,,	Considerable reduction of patternless oedematous cortex	0·4
8	23	,,	Considerable reduction in cortex, pattern moderately clear	0·75
14	48	,,	Broad cortex with indistinct pattern	1·1
23	18	,,	Great reduction in completely patternless cortex	0·7
27	53		Great dilatation of pelvis with thinning of renal substance	0·85
29	20	,,	Marked narrowing of cortex. Amyloid infiltration	0·5
31	53	,,	Considerable reduction in width of cortex and greatly blurred pattern	0·45
A 257	46	,,	Typical red granular kidney. Moderate reduction in cortex. Granular distorted pattern	1·0

CXLVIII. KIDNEY PHOSPHATASE.

II. THE ENZYME IN DISEASE.

By REGINALD THOMAS BRAIN
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(*Received August 24th, 1927.*)

In an investigation recently described by one of us in this *Journal* [Kay, 1926] data were quoted which strongly suggested a correlation between the phosphatase content of the kidney and the functional activity of this organ.

Further support for this suggestion has now been obtained from a study of the phosphatase content of (*a*) human kidneys, obtained *post mortem* from individuals dying from various causes, including kidney disease, (*b*) kidneys of rabbits with acute experimental nephritis.

METHOD.

The method used for determining the phosphatase content of a given kidney is that described on pp. 795 and 797 of the communication just mentioned. The activity of the phosphatase of rabbit's, rat's and pig's kidney has been previously shown to have a fairly broad optimum between p_H 8·8 and 9·2. This is true also for human kidney phosphatase. The optimum reaction becomes somewhat less alkaline as the period over which the activity of the enzyme is determined increases. In the great majority of the determinations which are given in the present paper, the activity of each kidney extract has been ascertained at a series of p_H values from 8·25 to 10·0. For two hours' activity the optimum is invariably between p_H 8·8 and 9·2. The unit of kidney phosphatase we define as the amount of enzyme which, at 38°, and in Sørensen's glycine-NaOH buffer solution at p_H 8·9, is required to liberate 1 mg. phosphorus as inorganic phosphate from excess of sodium β-glycerophosphate solution in 2 hours. The phosphatase activity of a tissue we express in units per g. (wet weight). Provided care is exercised in the sampling of the tissue (which should be obtained with as little delay as possible *post mortem*), analyses by different observers on the same kidney give reasonably concordant results (Table 1).

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29	20	,,	Marked narrowing of cortex. Amyloid infiltration	0·5
31	53	,,	Considerable reduction in width of cortex and greatly blurred pattern	0·45
A 257	46	,,	Typical red granular kidney. Moderate reduction in cortex. Granular distorted pattern	1·0

Table IV. *Cases in which death was due to causes other than nephritis, but in which renal abnormalities were found on* post mortem *examination.*

No. of expt.	Age of individual	Cause of death	Report on kidneys	Phosphatase activity
4	75	Broncho-pneumonia	Atrophied kidneys with a few cysts in cortex	2·2
21	53	Cerebral tumour	Bilateral cystic kidney with cysts throughout cortex and medulla	1·0
7	25	Heart failure	A few cysts and small areas of infarction in cortex beneath the capsule	2·9
30	63	Strangulated hernia. General peritonitis	Slight sub-capsular fibrosis and a few cysts	1·4

Table V. *Cases in which a severe infection was present before death.*

No. of expt.	Age of individual	Description of case	Report on kidneys	Phosphatase activity
11	43	Post-operative broncho-pneumonia	Severe parenchy. degeneration and oedema	4·6
13	50	Pericarditis. Heart failure	Congestion	4·7
15	22	Broncho-pneumonia	Parenchy. degeneration and oedema	2·8
19	14	Lobar pneumonia	Severe parenchy. degeneration	5·7
16	51	Pernicious anaemia. Tuberculosis	Anaemia, parenchy. degeneration and miliary tubercles	3·4
22	41	Broncho-pneumonia. Peritonitis	Anaemia, parenchy. degeneration	2·8
12	46	Hydatid disease of the liver. General peritonitis	Fatty or lipoid degeneration	2·25

Table VI. *Average values.*

Description of cases	Ages	No. of cases	Average value for kidney phosphatase
Normal	2 days and under	5	1·6
Normal, or no evidence of renal disease	3 mths. to 60 yrs.	11	4·8
Chronic nephritics	18 yrs. to 53 yrs	9	0·8
Not nephritic but other renal abnormality	25 yrs. to 75 yrs.	4	1·9
Severe infective conditions	14 yrs. to 51 yrs.	7	3·7

Table VI summarises the first series of findings:

(*a*) Foetal and very young kidney tissue is considerably less active than older tissue.

(*b*) In patients with chronic nephritis dying from uraemia, the phosphatase content of the kidney is markedly diminished, being reduced, on the average, to about one-sixth of the normal adult value.

(*c*) In cases in which death was not due to nephritis, but in which degenerative conditions were found in the kidneys *post mortem*, the phosphatase content of the kidney is also much reduced.

(*d*) The kidneys from cases in which a severe infective process was present before death, show some diminution in phosphatase content which is, however, much less marked than in (*b*) or (*c*) above.

B. KIDNEYS OF RABBITS WITH ACUTE EXPERIMENTAL NEPHRITIS.

Acute experimental nephritis was induced in rabbits by subcutaneous or intravenous injection of lethal doses (from 1·3 to 4 mg. U per kg.) of uranyl nitrate [cf. MacNider, 1920]. Death usually ensued from 4 to 7 days after the injection, and the kidneys were examined as soon as possible *post mortem*. Together with cardiac dilatation, there was invariably severe generalised oedema with large exudates of clear fluid into the peritoneal and pleural cavities. The kidneys were swollen and oedematous, but there was little if any change in the relative breadth of the cortex to that of the medulla.

In addition to the phosphatase content, the ratio dry weight to wet weight was determined in a number of cases, in order that the numerical correction of the phosphatase content for the "dilution" of the kidney tissue with oedema fluid might be ascertained.

The results are shown in Table VII.

Table VII.

Kidneys of control rabbits			Kidneys of animals with experimental nephritis				
No. of expt.	% dry wt.	Phosphatase activity	No. of expt.		% dry wt.	Phosphatase activity	Summary of histological report on kidneys*
219 (1 animal)	—	8·1	219 (2 animals)	A	—	2·9 L.	In all cases a severe degeneration of the tubules, and to a less extent of the glomeruli was found. In 219 B glomerular damage was relatively trivial. The order of severity of damage in these four animals based on histological examination was as follows: (1) 221 A (R) (2) 221 B (R) (3) 219 A (R) (4) 219 B (R)
					—	3·1 R.	
				B	—	3·3 L.	
					—	3·7 R.	
221 (2 animals)	23·9	10·9 L.	221 (2 animals)	A	17·5	2·4 R.	
	22·9	12·5 R.			17·6	2·3 L.	
	24·0	12·6 L.		B	16·1	2·5 R.	
					15·6	2·2 L.	
228 (1 animal)	23·8	10·7 L.	228 (1 animal)		15·7	2·8 L.	Severe degeneration and necrosis of the tubules
	22·4	-- R.			16·0	3·3 R.	
238 (1 animal)	—	12·6	238 (1 animal)		—	3·4 L.	Not examined
					—	3·4 R.	

L. = left kidney; R. = right kidney.

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* We are much indebted to Dr D. S. Russell for her kindness in making a histological examination of these kidneys.

From Tables VI and VII it will be seen that severe kidney damage, whether brought about by a protracted process of disease or induced rapidly by a toxic agent, is associated with a very large fall in the phosphatase content of the tissue. The observed diminution far outweighs any decrease which the "dilution" of the tissue by oedema fluid might bring about. Thus taking Exp. 221, Table VII, the average dry weight of the normal kidney was 23·6 % of the wet weight, giving an average phosphatase content of 50·5 units per g. dry weight. For the nephritic kidneys, with an average dry weight of 16·7 % of the wet weight, the phosphatase content per g. dry weight is only 23·5 units.

From Table VII it will also be seen that the order of increasing severity of damage, as determined histologically by a second observer, agrees with the order of decreasing phosphatase content per g. of tissue.

In the previous communication, already cited, we have shown that in the developing animal the phosphatase content of kidney tissue increases with increasing functional activity of this organ. Also, the renal cortex, which contains the essential secretory elements of the kidney, was shown to contain more phosphatase than the medulla. In the present paper, a diminished phosphatase content of the tissue is shown to be associated with diminishing functional capacity resultant from disease. These results suggest that the enzyme phosphatase plays an intimate part in renal activity, either cellular or secretory, and that its presence in amounts greater than a certain minimum is essential for the proper functioning of the organ.

It is admitted that the evidence is circumstantial and indirect, and that we may be dealing here merely with an associated failure of actually independent variables. Thus (*a*) in chronic nephritis the observed diminution in phosphatase activity may be due to the progressive decrease in the number of secretory units and their replacement by scar tissue, leading to a relative and absolute diminution in the amount of the more active cortical tissue; (*b*) in acute experimental nephritis, the poisoning and subsequent destruction of the glomerular and tubule cells would naturally result in the loss of many activities associated with the healthy cells (*e.g.* oxygen uptake, lipase and ereptase activity, glycolytic power), without there being of necessity any direct connection between the activities concerned—in this case between phosphatase activity and secretory ability. Nevertheless, the considerable circumstantial evidence in favour of a more direct relationship between the last two activities is at least not weakened by the results just described.

SUMMARY.

Both in chronic nephritis in man and in acute experimental nephritis in rabbits, the phosphatase activity of the renal tissue is markedly reduced.

The work has been carried out with the aid of a grant from the Research Fund of the London Hospital Medical College.

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CXLIX. IDENTITY OF URINARY ALBUMIN.

By LESLIE FRANK HEWITT.

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(*Received August 27th, 1927.*)

In general albuminuria is accompanied by a decrease in the proportion of albumin in the blood, and the object of this investigation is to determine whether blood-albumin passes through the kidney unchanged or if there is a difference between blood- and urinary albumin.

It was shown in a recent paper [Hewitt, 1927] that proteins when purified possess a characteristic optical rotatory power and dispersion, and this provides a sensitive method for their identification. Hynd [1925] has examined albumins isolated from urine but the figures obtained for the specific rotatory powers of the specimens vary within very wide limits, probably due to non-removal of lipins and other substances which seriously affect the rotation.

Albumins excreted by nephritic patients have been isolated from urine, purified, and their rotatory power and dispersion determined.

Isolation and purification of urinary albumins.

Urine was collected in bottles containing toluene and worked up as soon after collection as possible. In some experiments the albuminous urines were first concentrated by ultrafiltration. For this purpose flat collodion discs were used, mounted with rubber washers in a large Seitz filter. The membranes were prepared by pouring a filtered 6 % solution of pyroxylin in a mixture of alcohol (50 vols.), ether (50 vols.) and acetic acid (10 vols.) on a flat glass plate. After leaving for a few minutes for the ether to evaporate, the glass plate was plunged into cold water, when the membrane became readily detachable. After washing thoroughly in water, the membrane was cut to shape and fixed in the Seitz filter. If the membranes were allowed to dry, they shrank and became impermeable. Experiments with samples of cellophane kindly supplied by the British Cellophane Company were not successful, filtration being much too slow. Negative pressure was applied to the filtrate side of the apparatus and a positive pressure up to 10 atmospheres was applied to the other side using a reservoir constructed by Mr Macarthy in this Hospital.

To the filtered (or concentrated) urine saturated ammonium sulphate solution was added to make the mixture at least 60 % saturated with ammonium sulphate and acetic acid was added until the reaction was about p_H 4·7. After allowing to stand the precipitated proteins were filtered off on a Büchner funnel, dissolved in a small quantity of water, the solution neutralised, and cooled to 0° and then added drop by drop to about 8 times its volume of a mixture of alcohol (7 parts) and ether (3 parts) kept at − 15°. After standing at low temperature the precipitated proteins were filtered on a chilled Büchner funnel in the ice-chest, thoroughly washed with cooled alcohol-ether mixture, and with cold ether, and then rapidly transferred to a Soxhlet thimble and extracted for 20 hours with ether, metallic sodium being placed in the extraction flask to prevent any alcohol present from vaporising. After the first two hours' extraction, the ether and sodium were renewed. The extracted proteins were transferred to a vacuum desiccator and dried *in vacuo* over sulphuric acid for 48 hours, and then allowed to remain exposed to laboratory air for 24 hours, when the powdery solid was no longer readily electrified and was more easily "wetted," and hence dissolved in water more rapidly. The solid was pulverised, dissolved in water, any insoluble material (*e.g.* uric acid) filtered off or removed by centrifuging, and the solution made half saturated with ammonium sulphate by the addition of saturated ammonium sulphate solution. After allowing to stand overnight the precipitated globulins were filtered off, and the albumins precipitated in the mother-liquor by adding a further quantity of saturated ammonium sulphate solution and adjusting the reaction to about p_H 4·8 by adding 10 % acetic acid. After allowing to remain overnight the albumin was filtered off, dissolved in water, the solution neutralised and the above precipitation repeated. After one further repetition of the process the albumin was dissolved in a small quantity of distilled water, the solution placed in a collodion sac and dialysed against distilled water which was renewed frequently for at least 10 days. The clear dialysed solution was used for optical work.

Optical rotatory power and dispersion of urinary albumins.

The optical rotations of the solutions of the albumins obtained as described were determined in 1 dm. tubes, a Schmidt and Haentzsch polarimeter being used. The amount of protein present in the solutions was determined by Dr J. R. Marrack using the Kjeldahl method. In the following table specific rotatory powers for the mercury green line (λ = 5461 Å.) are quoted.

Patient	$[\alpha]_{5461}$	Diagnosis
1	−76·9°	Chronic nephritis with oedema
2 *a*	−78·9	Chronic nephritis with oedema
2 *b*	−76·9	
2 *c*	−79·6	
3	−78·0	Subacute chronic nephritis, oedema and uraemia
4	−74·8	Chronic nephritis, oedema and uraemia
5	−77·5	Albuminuria of pregnancy; chronic nephritis
6	−77·3	Albuminuria of pregnancy
Urinary albumin (mean)	−77·6	
Serum-albumin [Hewitt, 1927]	−78·1	

It will be seen that the mean values for urinary albumin (− 77·6°) and serum albumin (− 78·1°) are, within the limits of error, identical, so that it is probable that urinary and serum-albumins are the same protein. The specific rotatory powers of the albumins were determined also for light of different wave lengths. The results were

Albumin	λ - 6660	5780	5461	4358 Å.
Urinary (mean of 8)	−47 7°	−67·3°	−77·6°	−148·1°
Serum	−47·3°	−67·0°	−78·1°	−151·5°

The rather low figure $[\alpha]_{4358} - 148{\cdot}1^\circ$ for urinary albumin can be explained, since some of the specimens were somewhat dark in colour and absorbed a considerable amount of blue light, rendering accurate measurements impossible. Apart from this, very close agreement is observed. It is therefore rendered extremely probable that serum-albumin passes through the kidney unchanged.

SUMMARY.

The albumins obtained from the urine of patients with chronic nephritis and albuminuria of pregnancy possess the same optical rotatory power and dispersion as serum-albumin, and are therefore probably identical with it.

The author's thanks are due to Dr J. R. Marrack for his helpful interest in this work, which was carried out under the auspices of the Freedom Research Fund.

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CL. THE ETHER-SOLUBLE SUBSTANCES OF CABBAGE LEAF CYTOPLASM.

IV. FURTHER OBSERVATIONS ON DIGLYCERIDEPHOSPHORIC ACID.

By HAROLD JOHN CHANNON
and ALBERT CHARLES CHIBNALL.

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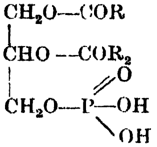
(*Received August 29th, 1927.*)

In a previous paper [Chibnall and Channon, 1927] it was shown that the ether extract of cabbage leaf cytoplasm contains very little, if any, phospholipin, in the commonly accepted use of this term. The major part of the ether-soluble phosphorus was present as the calcium salt of a diglyceridephosphoric acid of the type



Sufficient evidence was adduced to substantiate this structural formula, but the small amount of material then at our disposal did not enable us to obtain all the information which we considered was necessary for establishing the relationship of this acid to the two commoner phospholipins, lecithin and kephalin.

During the present summer we have prepared nearly 60 g. of the crude calcium salt from about 220 kg. of cabbage leaves, and have been able to investigate further two of the questions that had perforce been left open.

(1) In all our earlier work on this diglyceridephosphoric acid we had been troubled by the persistence with which small amounts of iron and nitrogen clung to what were considered purified products. We have now prepared this acid free from iron by dissolving it in a small volume of cold absolute alcohol. There remains undissolved a substance containing 8·9 % of iron and no nitrogen, which is extremely soluble in ether and is completely precipitated from ethereal solution by the addition of only one volume of alcohol. This explains why our previous efforts to purify the diglyceridephosphoric acid

by precipitation of its lead salt from ethereal solution by alcohol were unsuccessful. The yield of this unknown iron-containing substance is extremely small, 1·1 g. from 220 kg. of leaves. In view of the known connection in chlorotic leaves between chlorophyll synthesis and iron, we are undertaking a further investigation of this substance.

Although we have thus prepared the diglyceridephosphoric acid free from iron, we are still unable to remove the small amount of nitrogen (0·11 %). As to the nature of the substance giving rise to this nitrogen, we have no information. The natural suggestion would be that lecithin or kephalin was responsible for it. From the method of preparation, it seems improbable that the nitrogen was due to the presence of lecithin. In the preparation of the diglyceridephosphoric acid, the material precipitated from ether by acetone was repeatedly extracted with boiling acetone to remove certain other substances, the nature of which will be subsequently discussed. This treatment would tend to remove lecithin in the small amounts in which it could be present, for lecithin has a definite solubility in that solvent. Further, in this and previous work, we have purified the diglyceridephosphoric acid by repeated precipitation of its lead salt from ether solution by alcohol. This treatment would certainly have removed lecithin, and it seems probable that the small amount of kephalin which would be necessary to give rise to this percentage of nitrogen would have been removed also. The diglyceridephosphoric acid is also very soluble in cold acetone. In addition, as was previously reported, we have been unable to obtain at any time a precipitate with alcoholic cadmium chloride, and the nitrogen after the baryta hydrolysis in the preparation of the barium glycerophosphate remains insoluble in water. Hence we have no evidence whatever in favour of this 0·1 % of nitrogen being due to the presence of lecithin or kephalin, and it seems probable that there is some other ether-soluble nitrogen-containing substance present. This question must remain open until material is available in greater bulk.

(2) The nature of the fatty acids resulting from hydrolysis of the diglyceridephosphoric acid required more detailed investigation. We have now been able to show that the saturated acids are probably stearic and palmitic and that the unsaturated acids are linolic and linolenic. Oleic acid has not been identified, though that acid may be present in small amounts. A distinguishing feature is the low proportion of saturated acids. Levene, in his papers on lecithins and kephalins of animal origin, has shown that the acids consist of equal molecular proportions of saturated and unsaturated acids. But his more recent work on soya bean phosphatides has shown that this relationship may not hold in the plant, for his soya bean lecithin contained a low proportion of saturated acids. This is likewise the case with the diglyceridephosphoric acid. In the first place, the high degree of unsaturation of the mixed acids (iodine value, 143) is greater than that found in the acids from liver lecithins, which are characterised by the presence of arachidonic acid, which has four double bonds. We have found no trace of an acid more un-

saturated than linolenic in the mixed acids under discussion. In the second place, the fractionation of 13·2 g. of the mixed acids by means of the lead soaps yielded 2·85 g. of solid acids of iodine value 44, and 9·35 g. of liquid acids of iodine value 173. The major part of the latter fraction must have been linolic acid, for only 2·1 g. of hexabromostearic acid, equivalent to 0·8 g. of linolenic acid, were subsequently isolated from it. Therefore, the amount of saturated acids remaining in the liquid acids must have been small, and there is no doubt that the proportion of saturated acids in the original mixed acids was considerably less than one half.

The further evidence submitted in the present paper confirms our original contention that the free acid has the structural formula already given. It is therefore necessary to consider in some detail the class of substances to which this free acid and its salts are to be assigned. The free acid is an oil which is freely soluble in organic solvents, but has a very low solubility in water. The sodium salt is insoluble in ether, has a low solubility in cold alcohol and is soluble in water. The barium, calcium and lead salts are plastic solids which are insoluble in water, very readily soluble in ether, and are precipitated from ethereal solution by both acetone and alcohol. These properties, together with the fact that the acid and its salts readily darken on exposure to air, are essentially those of kephalin.

It is to be remembered that Thudichum divided the substances which give rise on hydrolysis to fatty acids, nitrogen and phosphorus, into two classes, the phosphatides and the cerebrosides. Leathes [1910] put forward reasons for altering these terms to phospholipin and galactolipin respectively. Thudichum's classification [1884] was based primarily on whether or not the substance contained phosphorus. His cerebrosides were nitrogen-containing but phosphorus-free fatty substances, while his phosphatides, as their generic name indicates, contained phosphorus but did not necessarily contain nitrogen. Thus, although his kephalophosphoric acid, the existence of which has yet to be proved, was nitrogen-free, it was classed as a phosphatide.

In his discussion of Leathes' nomenclature, MacLean [1918] defines the term lipins as "substances of a fat-like nature yielding on hydrolysis fatty acids or derivatives of fatty acids, and containing in their molecule either nitrogen or nitrogen and phosphorus." The essential point of the classification of Leathes is thus the nitrogen content of the substance, and from this point of view it seems to us the less satisfactory. Since Thudichum's classification is based on the phosphorus content of the fat, *i.e.* phosphatides are "phosphorised" fats, whether they contain nitrogen or not, the substance discussed in this paper readily finds a place in it, while it remains outside that of Leathes. There is the further point that the terms phosphatide and cerebroside have been in international use since 1880 and are in use to-day. They are wider terms and can be used to embrace a greater number of similar substances than those suggested by Leathes, and until research has further elucidated the difficult question as to what further compounds of these types exist, it seems to us preferable to keep to Thudichum's original definitions. If this be done the diglyceridephosphoric acid and its salts are phosphatides. Since the acid is chemically the parent acid of the two more commonly occurring phosphatides lecithin and kephalin, we propose to call it *phosphatidic acid* and the calcium salt found by us in the plant becomes calcium phosphatidate.

As the latter may thus be regarded as the simplest of the phosphatides so far reported, the question arises as to whether it may not be the precursor of lecithin and kephalin in the animal body. It is to be remembered that kephalin preparations have been reported to be contaminated by calcium. Thus Diaconow [1867] reported that the kephalin fraction (ether-soluble, alcohol-insoluble) from eggs contained calcium which can be removed by shaking its ethereal solution with HCl, and that the acid solution contained no phosphoric acid, showing that the calcium was not present in inorganic combination. Thudichum reported that after treatment of an ethereal solution of kephalin with HCl, the latter on evaporation yielded an ash containing calcium and iron. Parnas [1909] made similar observations and stated that an ethereal solution of crude kephalin loses its fluorescence when it is shaken with HCl. This fluorescence returns if the calcium-free ether solution is shaken with lime water. There would thus seem to be sufficient evidence that calcium in chemical combination with organic material in a form which is soluble in ether does exist in those tissues from which phosphatide preparations are usually made. This ether-soluble calcium appears in the kephalin fraction and can be removed by simple shaking with dilute mineral acid. In other words, it has similar solubility properties and is acted on by mineral acid in the same way as calcium phosphatidate. Further, Levene and Komatsu [1919], in their studies on cuorin, found that by careful fractionation of the phosphatide preparation from heart muscle they were able to obtain fractions which were beginning to approximate to various simpler kephalin derivatives, such as a monoglyceridephosphoric acid, etc. These may be decomposition products and this view is the one usually adopted; but there seems to us no sufficient reason why we should expect to find only pure kephalin and lecithin in such a tissue as, say, heart muscle. Because these phosphatides are extremely difficult to prepare in an analytically pure state, and because of the great ease with which they are oxidised, the conception has arisen that every so-called impurity in such preparations is the result of decomposition of the material through chemical manipulation or enzyme action. May not the alternative view be the more probable, namely, that these simpler products are normally present to some extent in such tissues, and that their presence is the cause of the variation in the N : P ratios on which the existence of so many phosphatides has been postulated, only to be overthrown on more careful examination?

Work is in progress with a view to obtaining further information as to the possible occurrence of nitrogen-free phosphatidic substances in animal tissues. Certain of the difficulties which will arise are obvious, not the least of them being the necessity of ensuring that any such substance isolated has not arisen through the decomposition of the primary substances present. Recently a paper has appeared [Wadsworth, Maltaner and Maltaner, 1927], in which the authors claim to have obtained a nitrogen-free phosphatidic substance from heart muscle, after previous treatment of an extract with ammonia and calcium chloride. To this substance they attribute the structure of calcium phosphatidate. The chemical evidence which these authors have so far produced is wholly unsatisfactory. We shall defer detailed criticism of this paper until the results of our investigations on heart muscle, which are among the tissues which we have been studying for some time from this point of view, are more complete.

In discussing the analytical data which follow, we have continued to use the figures for distearylglycerophosphoric acid as a basis for theoretical calculation.

EXPERIMENTAL.

Preparation of the crude calcium salt.

220 kg. leaves from winter-sown cabbage picked in May were treated as described in the earlier paper. 220 g. ether-soluble substances were extracted, from which 58 g. crude calcium salt were obtained after precipitation with acetone and extraction of the insoluble material with hot acetone.

Crude calcium salt R.

0·1008 g. substance: 0·2077 g. CO_2, 0·0763 g. H_2O, 0·0222 g. ash.
0·2903 g. substance: 0·0043 g. Fe_2O_3 and 0·0506 g. $Mg_2P_2O_7$.
0·2902 g. substance: 0·0628 g. $CaSO_4$.
0·2903 g. substance: 0·0632 g. AgI.
0·1160 g. substance required (micro-Kjeldahl) 2·0 cc. 0·01 *N* acid.

	C	H	Ca	P	Fe	Ash	Glycerol	N
Found	56·19	8·40	6·37	4·86	1·00	22·03	8·54	0·24 %.

2·90 g. on saponification yielded 1·77 g. of fatty acids or 61·1 % and 0·0512 g. of unsaponifiable matter or 1·8 %.

Isolation of phosphatidic acid.

50 g. crude calcium salt in 800 cc. ether were shaken once with 400 cc. *N*/2 H_2SO_4 and four times successively with 200 cc. *N*/4 H_2SO_4 to remove the calcium. The ether solution of the free acids was shaken four times with an aqueous solution of lead acetate, the ether solution of lead salts washed twice with water, and the solution concentrated to 300 cc. *in vacuo*. Two volumes of absolute alcohol were then added and the precipitated lead salts filtered off. These were dissolved in 200 cc. ether and reprecipitated with alcohol: yield, 36·0 g.

Lead salt of phosphatidic acid R.

0·0907 g. substance: 0·1675 g. CO_2, 0·0405 g. H_2O, 0·0288 g. ash.

	C	H	Ash
Found	50·36	7·41	31·75 %
Theory	51·47	8·14	32·30 %

When concentrated *in vacuo* the alcohol-ether mother-liquors gave a further precipitate of lead salt weighing 1·4 g., making a gross yield of 37·4 g. 34·9 g. of this lead salt in 900 cc. ether were shaken four times with *N*/4 HCl to remove the lead, washed four times with water and concentrated *in vacuo* to a syrup. 300 cc. cold absolute alcohol were then added to dissolve the phosphatidic acid. The insoluble material was removed by centrifuging, dissolved in ether and again shaken with *N*/4 HCl as before. A small amount of lead as chloride was removed, and, after washing, the ethereal solution was concentrated to 20 cc. *in vacuo*. Two volumes of alcohol were added with stirring, when the precipitated material collected as a brown gummy mass around the stirring rod. The yield of this insoluble material, which is the iron-containing material referred to in the introduction, was 1·1 g. The alcoholic solution on evaporation yielded 24·7 g. of phosphatidic acid. It is a pale olive-brown oil which flows slowly at room temperature, and clings tenaciously to traces of organic solvents. For analysis it was dried at 100° *in vacuo* for one hour, a treatment which does not affect its solubility in ether.

Phosphatidic acid R.

0·0771 g. substance: 0·1795 g. CO_2, 0·0663 g. H_2O.
0·2346 g. substance: 0·0374 g. $Mg_2P_2O_7$.
0·2306 g. substance (Zeisel-Fanto): 0·0728 g. AgI.
0·0938 g. substance required (micro-Kjeldahl) 0·80 cc. 0·01 *N* acid.
0·4692 g. substance required for neutralisation 13·4 cc. 0·1 *N* NaOH.
0·0938 g. substance absorbed 0·1079 g. iodine (Wijs); iodine value 115.

	C	H	P	M.W. (dibasic acid)	Glycerol	N
Found	65·71	10·09	4·42	700	12·5	0·11 %
Theory	66·57	10·94	4·40	704	13·1	0·0 %

3·51 g. on saponification with alcoholic potash gave 2·76 g. fatty acids of iodine value 143 and neutralisation value 199. Yield of fatty acids 78·6 %, theory for C_{18} saturated acid 80·6 %.

The total yield of phosphatidic acid represents 50 % and of the lead salt 55 % of the total phosphorus present in the original crude calcium salt. In view of the inevitable losses it is probable that about 60 % of the original phosphorus was present as calcium phosphatidate. The nature of the other 40 % of the phosphorus needs further investigation.

Products of hydrolysis of phosphatidic acid.

1. *Glycerophosphoric acid.* 15 g. phosphatidic acid dissolved in a small volume of alcohol were emulsified with water and added slowly to 750 cc.

boiling water in which had been dissolved 15 g. barium hydroxide. After standing on the water-bath for half an hour the precipitated barium soaps were filtered off, ground up with a further 300 cc. of baryta and boiled for one hour. This operation was repeated. Excess of barium was removed from the combined filtrates with carbon dioxide, and the filtrate from the barium carbonate concentrated to 50 cc. Two volumes of alcohol were added and the precipitated barium glycerophosphate filtered off. It was redissolved in water and boiled with charcoal until the filtrate was water-clear. The optical rotation was

$$[\alpha]^{20}_{\text{Hg green}} = +1{\cdot}3° \; (l = 2, c = 3).$$

This rotation confirms our earlier finding, but, as we mentioned in the previous paper, we consider that Levene's lengthy lead treatment should be applied before the question of the true rotation of this substance can be answered. We have not yet accumulated sufficient material for this purpose.

2. *Saturated fatty acids.* The barium soaps from the above hydrolysis were converted into the free acids and saponified with alcoholic potash to make certain that all the phosphatidic acid had been decomposed. 10·7 g. fatty acids obtained in this way, together with 2·5 g. from a previous saponification, 13·2 g. in all, were converted into the lead soaps and extracted with ether. The ether-insoluble lead soaps yielded 2·85 g. solid acids, iodine value 44. From 2·47 g. of these acids 1·66 g. of crystalline fatty acids were prepared by fractionation from 95 % methyl alcohol. This material was further separated by means of absolute methyl alcohol into two fractions—*A*, weighing 0·80 g. and *B*, weighing 0·53 g. These analysed as follows:

A. 0·0702 g. substance: 0·1937 g. CO_2 and 0·0810 g. H_2O.
0·2855 g. substance required for neutralisation 10·55 cc. 0·1 *N* alcoholic NaOH.

B. 0·1039 g. substance: 0·2871 g. CO_2 and 0·1150 g. H_2O.
0·2351 g. substance required for neutralisation 3·47 cc. 0·25 *N* NaOH.

Calculated $C_{16}H_{32}O_2$: C, 74·92; H, 12·58; M.W. 256; M.P. 63–4°.
Calculated $C_{18}H_{36}O_2$: C, 75·93; H, 12·78; M.W. 284; M.P. 70–71°.
Found (*A*) C, 75·25; H, 12·82; M.W. 280; M.P. 57°.
(*B*) C, 75·37; H, 12·33; M.W. 271; M.P. 56°.

These results suggest that the saturated acids consist of about equal parts of stearic and palmitic acids.

3. *Unsaturated fatty acids.* The ether-soluble lead soaps yielded 9·35 g. of liquid acids, iodine value 173. An attempt was made to fractionate these acids as barium soaps by benzene containing 5 % of 95 % alcohol, but no insoluble fraction could be obtained. Prolonged centrifuging deposited a little slime which was not worth separating, showing that the original liquid acids contained a small residue of saturated acids and only a small amount, if any, of oleic acid. The free acids were regenerated from the barium soaps, and brominated in anhydrous ether at 0°. The ether-insoluble precipitate,

after well washing with ether to remove excess bromine, weighed 2·1 g. and analysed as follows:

0·1487 g. substance: 0·1582 g. CO_2 and 0·0560 g. H_2O.

0·0992 g. substance required (Stepanoff) 7·85 cc. $N/10$ thiocyanate.

Calculated $C_{18}H_{30}O_2Br_6$: C, 28·49; H, 3·99; Br, 63·26; M.P. = 180–1°.

Found C, 29·01; H, 4·18; Br, 63·23; M.P. = 180°.

The substance is hexabromostearic acid, showing the presence of linolenic acid in the original mixed fatty acids.

The brominated acids soluble in ether (12·7 g.) were concentrated *in vacuo* to a syrup and extracted with hot light petroleum. From the ether-light petroleum solution there were obtained by alternate concentration *in vacuo* and cooling crops of white crystalline material which together weighed 0·84 g. After recrystallisation from light petroleum, the material melted at 110–111°, and analysed as shown below. A sample recrystallised twice from ether melted at 113° and analysed as follows:

0·1058 g. substance: 0·1358 g. CO_2, 0·0560 g. H_2O.

0·1023 g. substance required (Stepanoff) 6·95 cc. $N/10$ thiocyanate.

Calculated $C_{18}H_{30}O_2Br_4$: C, 36·01; H. 5·38; Br, 53·28; M.P. 113–4°.

Found C, 35·80; H, 5·89; Br, 54·41; M.P. 113°.

The substance is tetrabromostearic acid, showing the presence of linolic acid in the original mixed fatty acids.

The ether-light petroleum mother-liquor contained 5·38 g. of acids containing 45·0 % Br, showing that a small amount of oleic acid may have been present in the original mixed fatty acids.

SUMMARY.

Further observations on the calcium salt of a diglyceridephosphoric acid prepared from cabbage leaf cytoplasm have been made.

Reasons are given for considering the free acids and its salts as phosphatides.

The properties of the acid and its salts are discussed: since the acid is chemically the parent acid of lecithin and kephalin, the name phosphatidic acid has been assigned to it.

Phosphatidic acid differs from lecithin and kephalin, in which saturated and unsaturated fatty acids are present in equimolecular proportions, in that the greater part of the fatty acids present in the molecule is unsaturated.

The saturated acids are palmitic and stearic; the unsaturated acids are linolic and linolenic; oleic acid has not been identified; arachidonic acid is absent.

The possible occurrence of calcium phosphatidate in animal tissues is briefly discussed.

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CLI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VI.

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REPORT TO THE MEDICAL RESEARCH COUNCIL.

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THE experiments described in this communication are in continuation of a previously reported investigation on the behaviour of some of the inorganic constituents of lemon juice in the process of fractionation [Daubney and Zilva, 1926]. Nickel, cobalt, boron, iodine and phosphorus were considered in the first part of this research. The study of manganese and of iron in this connection became complicated by the presence of traces of these elements in the reagents used in the various manipulations. It was found that the mere absence of manganese and iron at the end of a blank experiment was not a fair indication that the two elements if found in the final fraction did not originate, at least in part, from the reagents used. Their presence in such fractions could only be considered of any significance if reagents free from all traces of the elements in question were used throughout the procedure. With this aim in view we have succeeded in preparing all our necessary reagents entirely free from interfering impurities. Manganese, iron, phosphorus and sulphur were investigated.

MANGANESE.

The scheme of fractionation adopted in this and in the following experiments was based on the improved method recently described by one of us [Zilva, 1927], eliminating the use of basic lead acetate. The method, although different in some details, did not differ essentially from the one employed by Daubney and Zilva in the first part of this research. The lemon juice was treated with excess of calcium carbonate, allowed to stand for about an hour and filtered. The filtrate was then concentrated by distillation in a vacuum at 50° to about a fifth of its original volume and treated with about half of its original volume of absolute alcohol. After filtering off the precipitate and removing the alcohol under reduced pressure at 50° the solution was diluted to the original volume of the juice with distilled water. After this the solution was fractionated according to the improved method mentioned above. All the reagents were previously purified.

Calcium carbonate was prepared from purified calcium chloride and sodium carbonate. The reagents were crystallised twice from distilled water. The resulting precipitate was thoroughly washed with distilled water and dried. To demonstrate that the chalk was free from manganese, 50 g. of it, *i.e.* about the amount required to neutralise 500 cc. of lemon juice, was stirred into an excess of dilute sulphuric acid. After filtering off the calcium sulphate the filtrate was concentrated to small bulk and any further precipitate of calcium sulphate removed. The liquid was then evaporated to dryness in a silica crucible and the residue tested for manganese by the method described later. Manganese was found to be totally absent. Ordinary precipitated chalk tested in the same way was found to contain appreciable quantities of manganese. The lead acetate was crystallised three times from dilute acetic acid, drained on a Büchner funnel and dried in the air. The absence of manganese from this reagent was demonstrated in the following ways.

(a) 10 g. of the salt (the quantity required for the treatment of an equivalent to 500 cc. of lemon juice) was dissolved in distilled water to which a drop of acetic acid was added to ensure perfect solution. The lead was then precipitated by the addition of an excess of sulphuric acid and the filtrate was evaporated to dryness in a silica crucible. Manganese was found to be absent from the residue.

(b) 10 g. of the lead acetate was dissolved as in *(a)* and magnesium sulphate added in slight excess above the quantity required to precipitate the lead. The mixture was then treated with two volumes of absolute alcohol, filtered, and the filtrate concentrated at 50° at low pressure, and eventually evaporated to dryness in a silica crucible. Manganese was absent from the residue.

No manganese was found in the filtrate when a solution of magnesium sulphate containing the quantity of the salt used in the manipulation was precipitated by the addition of two volumes of absolute alcohol, and the salt was therefore considered free from manganese.

The ammonia used in this experiment was also found to be free from all traces of manganese.

Bertrand's [1911] method, modified to suit our conditions, was employed in the estimation of manganese. In this method the manganese is oxidised to permanganate and is estimated colorimetrically. The ash of the various residues was heated with a few drops of sulphuric acid to drive off any chlorides present and after cooling was taken up in about 5 cc. of distilled water acidified with 1 cc. of concentrated nitric acid. The solution was then transferred to a specially selected test-tube suitable for colorimetric work, 5 drops of 10 % silver nitrate and about 0·1 g. of potassium persulphate were added and the mixture was boiled until the maximum pink coloration developed. Before matching, the solution was made up with distilled water to 10 cc.

A freshly made-up solution of potassium permanganate in graded dilutions and contained in suitable test-tubes was used as a standard. Manganous sulphate, which when oxidised as above yields equivalent quantities of permanganate, produces a solution which very often does not maintain its colour for any length of time and therefore the direct use of permanganate is more convenient.

It is possible in this way to determine manganese quantitatively within the limits of 0·005 and 0·15 mg., although the highest degree of accuracy is obtained within the range of 0·01–0·10 mg. in 10 cc. Below 0·005 mg. the coloration is perceptible but quantitative differentiation is unreliable. Above 0·10 mg. the difference in colour also gradually becomes less marked and solutions very much above this concentration show a tendency not to retain the pink colour and to deposit manganese dioxide. Although the latter compound can be reoxidised the procedure is troublesome. The sample of nitric acid used by us showed traces of manganese, but as this was the only reagent contaminated with this element, blanks, containing the quantity of nitric acid used in the entire determination including the incineration, were employed. It is to be pointed out that in the oxidation of manganese salts with nitric acid and persulphate the use of too much or too little of the acid influences the stability of the pink coloration.

Five batches of lemon juice were fractionated, two of 250 cc. each and three of 500 cc. each. In none of these could any manganese be detected in the final fraction. The decitrated lemon juice, and the supernatant liquid from which the active fraction was precipitated, contained 0·01 mg. of manganese per 500 cc. of juice.

Table I. *Mg. of Mn in fractions from 250 cc. of tomato juice.*

	No addition of Mn			*0·02 mg. Mn as $MnSO_4$ added to supernatant liquid from neutral lead acetate ppt.*	
No. of batch	Supernatant liquid from neutral lead acetate ppt.	Supernatant liquid from basic ppt.	Basic precipitate	Supernatant liquid from neutral lead acetate ppt.	Basic precipitate
I	0·055	0·060	nil	0·080	nil
II	0·040	0·040	—	0·060	„
III	0·080	0·080	nil	0·090	„
IV	0·060	0·060	„	0·070	„
V	0·060	0·060	„	0·080	„
VI	0·060	0·055	„	0·075	„

Apart from the above experiments a preliminary investigation on tomato juice produced similarly negative results. This work was done before the new method of fractionation was introduced and consequently the fractionation was carried out by the basic lead acetate method. The latter reagent was obtained free from manganese by recrystallising a sample of ordinary basic lead acetate from a saturated boiling water solution. The non-active material was first removed from the tomato juice by precipitation with two volumes of concentrated alcohol followed after removal of the alcohol from the filtrate by treatment with purified neutral lead acetate. The fraction precipitated by the basic lead acetate was taken up in dilute acetic acid and decomposed with sulphuric acid. On removing the lead sulphate, the filtrate was evaporated, incinerated and tested for manganese as previously described.

In this experiment the batches were divided into two series, to one of which $MnSO_4$ equivalent to 0·02 mg. of Mn per 250 cc. was added to the supernatant liquid from the neutral lead precipitate, in order to see whether inorganic manganese would be precipitated under these conditions. As will be seen from Table I no manganese was found in any of the final fractions. Similar fractions from tomato juice (decomposed however with $MgSO_4$) were tested biologically on guinea-pigs and were found to be active.

IRON.

The reagents used in the production of the active fractions for the estimation of iron were purified as in the case of the manganese estimations.

The calcium chloride and the sodium carbonate used in the preparation of the calcium carbonate were found free from iron after several crystallisations. Similarly the recrystallised neutral lead acetate on being decomposed with sulphuric acid was found to contain no iron. Nor did the ammonia and magnesium sulphate show the presence of any iron. The nitric acid contained traces of iron. This was compensated for by using blanks in the estimations. The iron was determined colorimetrically as ferric ferrocyanide, ferric alum stabilised by the addition of a few drops of hydrochloric acid being used for the standards. To the solution contained in specially selected test-tubes, 1 cc. of 0·5 % potassium ferrocyanide and a drop of concentrated hydrochloric acid were added for the production of the Prussian blue and the contents brought up to 10 cc. The optimum range was found to be between 0·02 and 0·15 mg. per 10 cc. By observing these conditions a colour of uniform tint and requisite durability was obtained. The various fractions were prepared, incinerated as for the manganese estimations and taken up in hydrochloric acid. Since about 0·15–0·26 mg. of iron per equivalent of a litre of original juice was found to be present, it was thought of interest to study the behaviour of this residual iron on dialysis through collodion membranes soaked in 88 % and in 95 % alcohol respectively [Zilva and Miura, 1921; Connell and Zilva, 1924; Daubney and Zilva, 1926]. This was carried out in the way described by

Table II. *Mg. of Fe in undialysed and dialysed fractions. Calculated for* 1 *litre of lemon juice.*

No. of batch	Decitrated alcohol-treated lemon juice	Final fraction undialysed	Final fraction after dialysis through thimbles of alcohol index	
			88 %	95 %
I	2·4	0·16	0·16	0·12
II	1·0	0·20	0·08	nil
III	1·6	0·28	0·14	„
IV	0·6	0·20	0·04	„
V	1·0	0·16	0·16	„
VI	1·3	0·16	0·12	„
VII	—	0·24	0·16	„

Daubney and Zilva. Iron was estimated in 50 cc. of decitrated juice and in equivalents of 250 cc. of the other fractions which as before were all reduced to 10 cc. for the estimation. Table II gives a summary of the results calculated for an equivalent of 1 litre of juice.

It is seen that the iron passed through an 88 % thimble only to a small extent whilst, with the exception of one case, the 95 % thimbles permitted the entire passage of the iron. This will be discussed in a later section of the paper.

PHOSPHORUS.

The reagents used in the fractionation were purified as before. The calcium carbonate was tested in the following ways. (*a*) The requisite quantity of chalk was treated with sulphuric acid until slightly acid, filtered, and the filtrate concentrated to about 5 cc. After filtering off any further calcium sulphate which separated out during concentration, the solution was tested for total phosphorus by the Martland and Robison modification [1926] of the Briggs method. (*b*) In this case the calcium carbonate was decomposed with citric acid using a quantity of the acid which was approximately equivalent to that contained in 100 cc. of lemon juice. After neutralising the acid it was treated with two volumes of alcohol, filtered, and the alcohol removed by distillation from the filtrate which was concentrated to about 5 cc. and tested for phosphorus by the above method. No traces of phosphorus could be detected by either method. Ordinary samples of precipitated chalk treated as above showed a content of 0·1 mg. of phosphorus per 10 g. of calcium carbonate.

The neutral lead acetate was tested for phosphorus after being decomposed by sulphuretted hydrogen and was found to be free, as was also the ammonia.

Lemon juice was fractionated as before, except that the final fraction was decomposed by sulphuretted hydrogen instead of by magnesium sulphate. Fractions treated in this way, as previously pointed out by one of us, lose their activity. It was found, however, that the magnesium sulphate treatment owing to the almost unavoidable presence of small quantities of this salt in the final fraction, made the ignitions of the organic matter, as carried out in the above method, troublesome. This inactivation of the fraction would not of course interfere with the inorganic content of the fraction. The analysis of six batches of the pure fraction equivalent to 100 cc. yielded 0·12, 0·13, 0·13, 0·14, 0·12, 0·11 mg. respectively and, as will be seen from Table III, the phosphorus in the final fractions dialysed only to a very small extent through collodion thimbles of 88 % alcohol index, whilst the thimbles with a 95 % index permitted the entire phosphorus to diffuse in the course of three days.

Attempts to estimate how much of the phosphorus was inorganic failed, owing to the turbidity and to the modification in the tint obtained by adding the Briggs reagent before ignition.

It may however be noted that disodium hydrogen phosphate and potassium dihydrogen phosphate in equivalent dilutions dialysed in the same way as the phosphorus of the final fraction.

Table III. *Mg. of P in final fractions. Calculated for* 1 *litre of lemon juice.*

No. of batch	Final fraction undialysed	Final fraction after dialysis through thimbles of alcohol index	
		88 %	95 %
I	1·28	1·13	nil
II	1·22	0·97	„
III	1·37	0·96	„
IV	1·17	0·95	„
V	1·10	0·74	„
VI	1·14	0·92	„
VII	1·11	1·08	„
VIII	1·20	1·00	„

The figures for total phosphorus obtained in these experiments are rather lower than those previously obtained [Zilva, 1924; Daubney and Zilva, 1926], but this no doubt is due to the use of purified reagents.

SULPHUR.

In spite of the use of reagents free from sulphur, quantities of this element were found in the final fraction which, considering the nature of the investigation, were of a high order. These figures were obtained without incinerating the preparation by direct precipitation as barium sulphate out of the solution which was previously heated with hydrochloric acid in order to convert any "ethereal" into inorganic sulphur. In nine batches, equivalent to 1 litre of original juice, 2·0, 3·6, nil, nil, 0·8, 2·3, 0·9 mg. were found. The sulphur in the active fraction diffused only partly through an "88 %" thimble, but entirely through a "95 %" collodion thimble. In view of these high and variable results the further effort of determining the total sulphur was not considered justified.

CONCLUSIONS.

In the two sections of this enquiry the active fraction from lemon juice has been examined for iron, phosphorus, sulphur, iodine, manganese, nickel, cobalt and boron. The last four elements were found to be absent and consequently, if at all associated with the antiscorbutic activity, they must be so in quantities not detectable by our methods. Of those present, iodine may also be excluded since, as has been shown by Daubney and Zilva, no evidence could be obtained that any of it diffused through a collodion thimble with a 95 % alcohol index. Antiscorbutic solutions dialysed under the same conditions lose their activity. On the other hand the iron, phosphorus and sulphur of the active fraction behaved on dialysis in the same way as the active principle. One must however be very cautious in postulating a connection between them and the antiscorbutic activity. These inorganic constituents cannot be responsible for the activity *per se* as they are almost ubiquitous in animal and plant tissues, whilst the distribution of the antiscorbutic factor in these tissues is limited to certain sources only. If there be any connection, these elements must be either in physical or chemical association with some other substance or radicle or themselves be in a definite physical state responsible for their physiological function. Needless to say this investigation offers no indication which would justify such hypotheses. The possibility that the presence of iron, phosphorus and sulphur in the active fraction is of significance must therefore await consideration until our knowledge of the problem is more advanced.

SUMMARY.

The concentrated antiscorbutic fraction from lemon juice does not contain any manganese but contains traces of iron, phosphorus and sulphur. These elements of the active fraction behave on dialysis like the active principle.

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CLII. LIVER DIASTASE.

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In his paper on liver diastase, Eadie [1927] says, "Lesser...has advocated another view, viz. that the glycogen and amylase are, as it were, 'locked up' in different compartments of the cell. This explanation unfortunately adds little to our knowledge because it suggests no mechanism whereby the two are kept separate."

Unfortunately Eadie quotes my researches only to the year 1921. In 1923 I showed [Lesser and Zipf, 1923] that homologous alcohols in isocapillary concentrations increase sugar formation in the perfused frog's liver by approximately the same amount, and later [Lesser, 1925] that this effect occurs under reversible conditions. Finally it has been shown [Lesser, 1926] that the effect is also similar in a perfusion fluid buffered with carbon dioxide and bicarbonate at p_H 7·4 and 7·6.

On the basis of these findings I have concluded, in agreement with Warburg [1923], that a boundary surface-phenomenon is involved, and have assumed that approximately $\frac{2}{3}$ of the liver diastase is adsorbed on the surfaces of the cell and is therefore unable to act on the liver glycogen. Similar experiments with the isolated parotid gland of the mouse [Lesser, 1927] have led to precisely the same results. Eadie is therefore incorrect in stating that I have not suggested a mechanism whereby the diastase and glycogen are kept separate.

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CLIII. THE EFFECT OF HYDROGEN ION CONCENTRATION AND PROTEIN CONCENTRATION ON THE OSMOTIC PRESSURE OF SERUM-PROTEINS.

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(*Received August 16th, 1927.*)

SEVERAL workers have shown that the osmotic pressure of serum- or plasma-proteins, in solutions of increasing protein concentration, increases more rapidly than the protein concentration.

Verney [1926] has shown that this relation is such as would be found if the protein in solution occupied many times its dry volume. There are, however, other factors which will cause a deviation from a linear relation between observed osmotic pressure and protein concentration. Particularly, there is an excess of diffusible ions in the protein solution over that in the outside solution, due to the Donnan effect; this excess of diffusible ions would, if the solutions were ideal, have an osmotic pressure, which we will call the Donnan pressure, over and above that due to the protein itself; and this Donnan pressure would be proportional to the square of the protein concentration, when the salt concentration of the outside fluid and the reaction were fixed. As a consequence the observed osmotic pressure (y) and protein concentration (x), at any particular p_H, would be connected by an equation of the form

$$y = ax + bx^2 \qquad \ldots\ldots(1),$$

a parabola convex to the axis of x, very similar to the curves found by Mayrs [1926] and Verney [1926].

The term b in equation (1) would vary with the p_H, so that the observed osmotic pressure should also vary with the p_H. Mayrs however was unable to detect such variation.

We have therefore studied the effect of varying protein concentration and p_H on the observed osmotic pressure, with the idea that although the actual effect of the Donnan equilibrium would not agree with that calculated for ideal solutions, it would still be demonstrable, and with the hope of showing deviations from ideality which will affect the relation between the observed osmotic pressure and the protein concentration.

The excess of diffusible ions in the protein solution, and the osmotic pressure resulting therefrom.

Symbols.

[Pr] = concentration of protein in the protein solution in g. per 1000 g. H_2O.

$[Pr^-]$ = concentration of protein ions in the protein solution in millequivalents per 1000 g. H_2O.

[BPr] = concentration of base bound by protein in the protein solution in millequivalents per 1000 g. H_2O.

$[A_P^-]$ = concentration of diffusible anions in the protein solution in millequivalents per 1000 g. H_2O.

$[B_P^+]$ = concentration of diffusible kations in the protein solution in millequivalents per 1000 g. H_2O.

$[B_P]$ = concentration of total base in the protein solution in millequivalents per 1000 g. H_2O.

$[Cl_P]$ = concentration of chlorine in the protein solution in millequivalents per 1000 g. H_2O.

$[Cl_P^-]$ = concentration of chlorine ions in the protein solution in millequivalents per 1000 g. H_2O.

$[HCO_{3P}^-]$ = concentration of bicarbonate ions in the protein solution in millequivalents per 1000 g. H_2O.

$[A_E^-]$ = concentration of diffusible anions in the external solution in millequivalents per 1000 g. H_2O.

$[B_E^-]$ = concentration of diffusible kations in the external solution in milliequivalents per 1000 g. H_2O.

$\alpha_{A_P}, \alpha_{B_P}, \alpha_{Cl_P}$, etc. = the activities of the ions in the protein solution.

$\alpha_{A_E}, \alpha_{B_E}, \alpha_{Cl_E}$, etc. = the activities of the ions in the external solution.

$f_{A_P}, f_{B_P}, f_{Cl_P}$, etc. and $f_{A_E}, f_{B_E}, f_{Cl_E}$, etc. = the activity coefficients of the ions in the two solutions.

Then $$\alpha_{A_P} = f_{A_P}[A_P^-];\ \alpha_{A_E} = f_{A_E}[A_E^-], \text{ etc.} \qquad \ldots\ldots(2).$$

Let $$[Pr^-] = k\,[BPr].$$

Now $$[B_P^+] = [A_P^-] + [Pr^-] = [A_P^-] + k\,[BPr] \qquad \ldots\ldots(3),$$

and

$$f_{A_P}[A_P^-].f_{B_P}[B_P^+] = \alpha_{A_P}.\alpha_{B_P} = \alpha_{A_E}.\alpha_{B_E} = f_{A_E}[A_E^-].f_{B_E}[B_E^+] = f_{A_E}.f_{B_E}.[A_E^-]^2 \qquad \ldots\ldots(4).$$

$$\therefore\ f_{A_P}.f_{B_P}.[A_P^-]\{[A_P^-] + k\,[BPr]\} = f_{A_E}.f_{B_E}.[A_E^-]^2.$$

$$\therefore\ [A_P^-] = \left[\frac{f_{A_E}.f_{B_E}}{f_{A_P}.f_{B_P}}[A_E^-]^2 + \frac{k^2}{4}[BPr]^2\right]^{\frac{1}{2}} - \frac{k}{2}[BPr]$$

$$= \left(\frac{f_{A_E}.f_{B_E}}{f_{A_P}.f_{B_P}}\right)^{\frac{1}{2}}[A_E^-] + \frac{k^2}{8}\left(\frac{f_{A_P}.f_{B_P}}{f_{A_E}.f_{B_E}}\right)^{\frac{1}{2}}.\frac{[BPr]^2}{[A_E^-]} - \frac{k}{2}[BPr] \qquad \ldots\ldots(5).$$

If k [BPr] is small compared with $[A_E^-]$;

similarly $$[B_P^-] = \left(\frac{f_{A_E} \cdot f_{B_E}}{f_{A_P} \cdot f_{B_P}}\right)^{\frac{1}{2}} [A_E^-] + \frac{k^2}{8} \left(\frac{f_{A_P} \cdot f_{B_P}}{f_{A_E} \cdot f_{B_E}}\right)^{\frac{1}{2}} \frac{[\mathrm{BPr}]^2}{[A_E^-]} + \frac{k}{2} [\mathrm{BPr}] \quad \ldots(6).$$

$$\therefore\ [A_P^-] + [B_P^-] = 2 \left(\frac{f_{A_E} \cdot f_{B_E}}{f_{A_P} \cdot f_{B_P}}\right)^{\frac{1}{2}} [A_E^-] + \frac{k^2}{4} \left(\frac{f_{A_P} \cdot f_{B_P}}{f_{A_E} \cdot f_{B_E}}\right)^{\frac{1}{2}} \frac{[\mathrm{BPr}]^2}{[A_E^-]} \quad \ldots\ldots(7).$$

If the effect of the protein on the activity coefficients of the diffusible ions is negligible, this becomes

$$[A_P^-] + [B_P^-] = 2\,[A_E^-] + \frac{k^2}{4} \frac{[\mathrm{BPr}]^2}{[A_E^-]},$$

that is, the excess of diffusible ions in the protein solution equals

$$\frac{k^2}{4} \cdot \frac{[\mathrm{BPr}]^2}{[A_E^-]}.$$

The osmotic pressure at 37° due to this excess, when $[A_E^-] = 160$, will be

$$26 \times 0{\cdot}938 \times \frac{k^2}{4} \frac{[\mathrm{BPr}]^2}{[A_E^-]} \text{ cm. } H_2O.$$

The coefficient 0·938 is derived from Bronsted's [1921] formula

$$1 - \phi = a z_1 z_2 \sqrt{\mu} + \beta c,$$

and the values for the activity coefficients at this ionic strength from Scatchard [1925].

If we assume that the activity coefficient of the protein does not change with varying protein concentration, the observed osmotic pressure (π) at constant p_H will be given by the equation

$$\pi = 26{,}000 \frac{[\mathrm{Pr}]}{M} + 26 \times 0{\cdot}938 \times \frac{k^2}{4} \times \frac{[\mathrm{BPr}]^2}{160} \quad \ldots\ldots(9),$$

(where M is the molecular weight of the protein), which is a parabola convex to the [Pr] axis. Also, if the activity coefficient of the protein is not affected by change of hydrogen ion concentration, the observed osmotic pressure of a solution of constant protein concentration will rise with rising p_H by the amount,

$$\Delta\pi = \frac{26 \times 0{\cdot}938}{4 \times 160} \times k^2 \times 0{\cdot}097^2\, [\mathrm{Pr}]^2\, [(p_{H_2} - 5{\cdot}26)^2 - (p_{H_1} - 5{\cdot}26)^2] \quad \ldots\ldots(10)$$

using equation (11) (see p. 1135) for [BPr].

If $k = 1$, the second term in equation (9) is equal to 13·1 cm. when [Pr] = 70 and the p_H is 8·0; and $\Delta\pi$ in equation (10) is equal to 7·6 cm. when [Pr] = 70 and $p_{H_1} = 7{\cdot}0$, $p_{H_2} = 8{\cdot}0$; thus the effect of this unequal distribution of diffusible ions will be by no means negligible if the assumptions with regard to activity coefficients are correct and the protein salt is fully dissociated.

METHODS.

Horse serum was separated from blood obtained from the slaughter house and allowed to clot. Human serum was obtained by shaking blood from two normal males (J. M. and L. H.) with glass beads to remove fibrin and centrifuging. The human sera were obtained under sterile conditions and both horse and human sera were filtered through a Seitz filter before use. The lower concentrations of protein were obtained by dilution with 0·9 % sodium chloride solution.

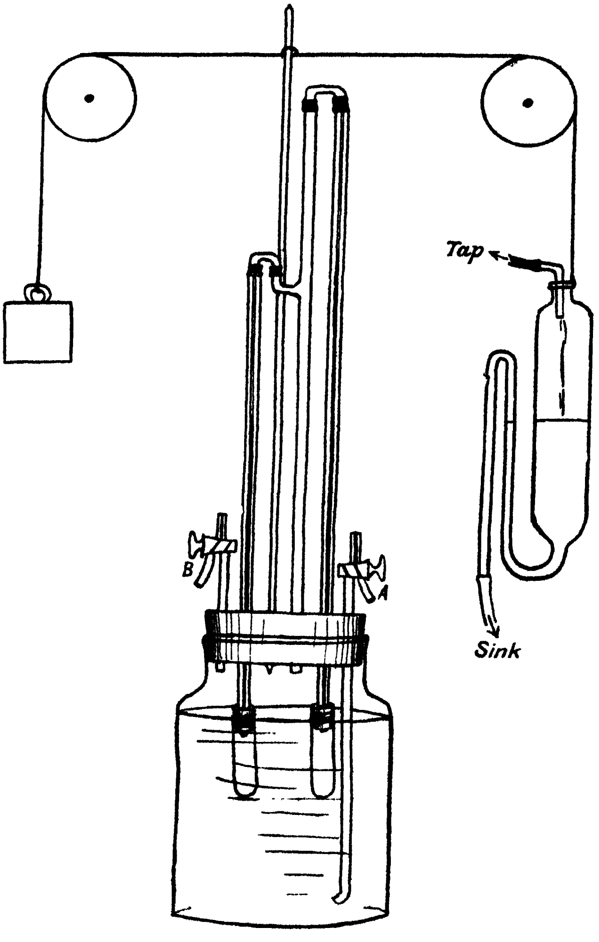

Fig. 1.

The osmotic pressures were measured with the apparatus shown in Fig. 1. The large bottle contained about 1 l. of a bicarbonate-carbon dioxide buffer mixture, containing phenolsulphonephthalein. The bicarbonate solution was first sterilised by boiling in a litre flask; CO_2 was then passed into it through a sterile cotton-wool filter, until the p_H was approximately that required. When necessary the p_H was adjusted more accurately after the solution had been placed in the bottle by running a CO_2-air mixture of known composition in through the tap *A* and out through *B*.

When the apparatus was in a water-bath the pressure inside the bottle was adjusted to atmospheric pressure by opening tap *B*.

Collodion sacs were made inside test-tubes using an 8 % solution of collodion (British Drug Houses) in a mixture of alcohol (50 volumes), ether (50 volumes) and glacial acetic acid (10 volumes). We are indebted to Mrs Dover who prepared many of the sacs used.

These sacs were attached to pressure tubing with rubber bands; they were made to hold about 8 cc. To check their impermeability to protein they were filled with serum, the rubber tubing was closed with a glass rod, and the sacs were placed for a night in normal saline, which was then tested with sulphosalicylic acid; if the faintest cloud appeared the sacs were rejected.

The sacs were found to be readily permeable to phenolsulphonephthalein; if they were filled with water and placed in a phenolsulphonephthalein solution over night, the concentrations of dye in the external and internal fluids were found to be the same next morning. The permeability was not altered by the process of sterilisation.

The capillary tubes had a bore of about 1 mm.; it was found that narrower tubes were unsatisfactory as the protein solution was liable to stick. Dilution of the protein solutions could be avoided by forcing the capillary tubes well into the collodion sacs so that the fluid rose in the capillary to about the height anticipated.

Those parts of the apparatus which could safely be heated were sterilised by autoclaving or by dry heat. The remainder, including the collodion membranes, were sterilised by soaking for 15 minutes with 70 % alcohol, which was washed off with sterile saline, or with alcohol and ether if the parts were required to be dry. The whole apparatus was assembled with aseptic precautions by an operator wearing sterile gloves.

These methods of sterilisation proved entirely satisfactory; even in subsequent experiments with egg-albumin we have had no bacterial contamination. The Seitz filters have proved most valuable when dealing with protein solutions which were not sterile to start with.

The apparatus was kept at 0° in a bath of ice and water for one day in order to let substances such as urea and electrolytes other than those present in the buffer solution dialyse out. The external fluid was then changed and the apparatus placed in a water-bath at 37° for 2 or more days. It was gently rocked and finally allowed to stand with the capillary tubes vertical; this final period without rocking was necessary, as during the short periods during which the bottle was tilted the vertical heights of the columns in the capillary tubes were reduced.

In the later experiments the gas pressure above the column of fluid was equal to that above the external fluid; in order to read the osmotic pressures it was only necessary to mark the heights of the columns of fluid in the capillary tubes, remove the apparatus from the water-bath and read the levels of these marks and of the surface of the external fluid with a vertical mirror scale.

In the earlier experiments the apparatus was slightly different. Taps were carried on the ends of the capillary tubes to which the collodion sacs were attached. These taps were kept closed during the dialysis. When an osmotic pressure was to be read the height of the column of fluid in a capillary was marked, and without removing the apparatus from the water-bath the tap was connected with a water manometer and opened. The manometer pressure was then adjusted until the surface of the protein solution in the capillary was the same as before the tap was opened; this gave the gas pressure above the column in the capillary tube. The height of the mark on the capillary tube was then measured in the same way as in the later experiments; the osmotic pressure was equal to the pressure of this column of fluid plus the manometer pressure. This method was much less satisfactory than the simpler method of the later experiments and the results are given only to show that even with less accurate methods fairly consistent results can be obtained.

In calculating the osmotic pressure, allowance was made for the specific gravity of the protein solutions, and for the rise due to surface tension.

After the osmotic pressures had been read, the apparatus was replaced in the water-bath to reheat (little cooling took place during the short time required for reading the heights of the columns) and a sample of gas was taken into a gas sampler full of mercury attached to tap *B*; clean mercury was run in by tap *A* at the same time to drive the gas out. Before this process dead spaces were filled with mercury. This gas sample was analysed in a Haldane apparatus.

A sample of the external fluid was then taken and the protein solutions were run into test-tubes with precautions to avoid letting the fluids in the capillaries run out, as these fluids might differ from those in the sacs.

The *total nitrogen* in the internal fluids was estimated by Kjeldahl's method; since other nitrogenous substances would have diffused away into the large volume of external fluid this nitrogen was assumed to be that of protein and the protein present was calculated, using 6·45 as the factor.

Chlorides were estimated by heating 1 cc. of fluid in a water-bath with excess of silver nitrate and nitric acid, and titrating the excess of silver nitrate with potassium thiosulphate, using iron alum as indicator. The error in this estimation was not more than 0·5 %.

Bicarbonates were estimated in the external fluid by boiling 20 cc. with a slight excess of *N*/10 HCl and titrating the excess with *N*/10 NaOH with methyl red as indicator. The bicarbonate concentrations in the protein solutions were calculated on the assumption that the ratio

$$\frac{\text{HCO}_3\text{ in protein solution}}{\text{HCO}_3\text{ in external solution}}\text{ was the same as }\frac{\text{Cl in protein solution}}{\text{Cl in external solution}}.$$

The p_H of the external fluid was calculated from the CO_2 tension and bicarbonate concentration by the formula

$$p_H = 6{\cdot}1 + \log [BHCO_3^-] - \log pCO_2 - \log 0{\cdot}0326.$$

The *total base* was estimated by the method of Stadie and Ross [1925]. The proteins of the protein solutions were first precipitated with trichloroacetic acid. In the external fluids the total base was found to be equivalent to the sum of the chloride and bicarbonate found, within 1 % in almost all cases.

In the estimations of chlorides and total base all quantities under 5 cc. were weighed, in order to avoid errors in the discharge of pipettes.

Specific gravities were estimated by weighing in a pyknometer; dry weights by drying a known volume to constant weight in an oven at 100°–110° and cooling in a desiccator.

In preliminary experiments it was found that appreciable loss of CO_2 by diffusion through the rubber stopper, and changes in alkalinity of the buffer mixture from solution of glass, did not occur.

Base bound by protein.

The values for [BPr] given in Table I are obtained by subtracting $[Cl\ ] + [HCO_{3p}]$ from the total $[B_p]$. The estimation by this means must be inaccurate as it is given by the small difference between two large quantities; nevertheless the results are fairly consistent. For human serum-proteins the results and their relation to p_H (Fig. 2) agree fairly well with the formula given by Hastings, Salvesen, Sendroy and Van Slyke [1927]

$$[BPr] = 0{\cdot}097\ [Pr]\ (p_H - 5{\cdot}26)\ \text{(curve A, Fig. 2)} \quad ......(11).$$

though they are slightly higher[1], but our results for horse serum-protein are consistently higher than those given by the formula of Van Slyke, Wu and MacLean [1923]

$$[BPr] = 0{\cdot}068\ [Pr]\ (p_H - 4{\cdot}80)\ \text{(curve B, Fig. 2)} \quad ......(12).$$

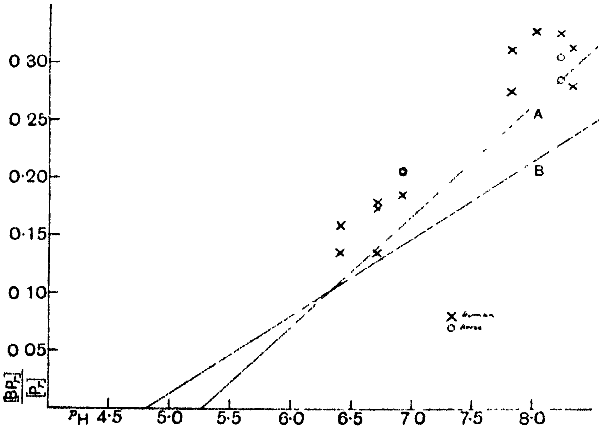

Fig. 2. Relation of base bound by protein to p_H.

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[1] The p_H in our calculations is that of the external fluid, not of the protein solution; the effect of this however is very small and in the opposite direction to the difference found.

Table I. *Distribution of* Cl, HCO_3 *and base.*

		External fluid				Protein solution								
Expt. No.	Serum	p_H	[Cl]	$[HCO_3]$	$[Cl]+[HCO_3]$	[Pr]	[Cl]	$[HCO_3]$	[B]	[BPr]	$\frac{[BPr]}{[Pr]}$	$\frac{[Cl_P]}{[Cl_E]}$	$\frac{[B_E]}{[B_P]}$	$\frac{[Cl_P][B_E]}{[Cl_E][B_P]}$
11 *b*	J. M.	6·4	142·0	—	—	57·2	142·3	—	—	—	—	1·002	—	—
5 *a*	,,	6·7	139·9	14·5	154·4	63·9	138·7	14·4	161·8	8·7	0·136	0·991	0·9545	0·963
5 *b*	,,	6·7	139·9	14·5	154·4	26·3	140·4	14·5	159·5	4·6	0·175	1·003	0·969	0·966
3 *a*	,,	6·9	144·9	15·0	159·9	50·4	143·7	14·9	168·0	9·4	0·187	0·9915	0·952	0·961
3 *b*	,,	6·9	144·9	15·0	159·9	34·5	145·0	15·0	—	—	—	1·001	—	—
12 *a*	,,	7·8	142·9	14·8	157·7	50·3	140·1	14·5	170·3	15·7	0·314	0·980	0·9265	0·943
13 *a*	,,	8·0	141·6	14·9	156·5	62·3	136·2	14·4	171·1	20·5	0·330	0·962	0·915	0·951
4 *a*	,,	8·3	139·9	14·5	154·4	67·8	135·1	14·0	167·9	18·8	0·282	0·965	0·9195	0·948
4 *b*	,,	8·3	139·9	14·5	154·4	37·1	137·1	14·2	163·2	11·9	0·315	0·987	0·946	0·965
6 *a*	,,	6·4	288·9	14·6	303·5	66·9	292·2	14·8	316·1	9·1	0·136	1·011	0·960	0·949
6 *b*	,,	6·4	288·9	14·6	303·5	36·7	292·3	14·8	—	—	—	1·021	—	—
7 *a*	,,	8·2	288·2	14·6	302·8	64·8	283·1	14·3	318·6	21·2	0·328	0·982	0·950	0·969
7 *b*	,,	8·2	288·2	14·6	302·8	32·2	287·0	14·5	—	—	—	0·996	—	—
11 *a*	L. H.	6·4	142·0	15·0	157·0	74·0	140·8	14·9	167·5	11·8	0·160	0·9915	0·9375	0·944
9 *a*	,,	6·7	142·6	15·0	157·6	67·6	139·7	14·7	166·6	12·2	0·180	0·9795	0·946	0·965
9 *b*	,,	6·7	142·6	15·0	157·6	40·9	141·5	14·9	—	—	—	0·992	—	—
10 *a*	,,	7·8	142·4	14·9	157·3	63·6	137·1	14·4	—	—	—	0·963	—	—
12 *b*	,,	7·8	142·9	14·8	157·7	68·6	138·2	14·3	171·5	19·0	0·277	0·967	0·920	0·949
1 *a*	Horse	6·9	125·4	30·6	156·0	70·5	122·0	29·7	166·4	14·7	0·208	0·971	0·9375	0·963
1 *b*	,,	6·9	125·4	30·6	156·0	42·5	124·0	30·2	163·0	8·8	0·207	0·989	0·957	0·966
2 *a*	,,	8·2	124·4	29·4	153·8	68·9	118·3	27·9	167·4	21·2	0·308	0·951	0·919	0·966
2 *b*	,,	8·2	124·4	29·4	153·8	42·6	121·6	28·7	162·5	12·2	0·286	0·9775	0·9465	0·969

Distribution of ions.

We find in all our experiments (Table II) that

$$\frac{[Cl_P]}{[Cl_E^-]} > \frac{[B_E^+]}{[B_P]} \qquad \text{......(13)},$$

or

$$\frac{[Cl_P]\,[B_P]}{[Cl_E^-]\,[B_P^-]} > 1 \qquad \text{......(13 } a)^1.$$

Now

$$\frac{f_{Cl_P}\,[Cl_P^-]}{f_{Cl_E}\,[Cl_E^-]} = \frac{\alpha_{Cl_P}}{\alpha_{Cl_E}} = \frac{\alpha_{B_E}}{\alpha_{B_P}} = \frac{f_{B_E}\,[B_E^+]}{f_{B_P}\,[B_P^+]} \qquad \text{......(14)}.$$

It follows that either $\frac{f_{Cl_P}}{f_{Cl_E}} < \frac{f_{B_E}}{f_{B_P}}$ or the ions form undissociated compounds with protein (making $[Cl_P^-] < [Cl_P]$ or $[B_P^+] < [B_P]$).

Our experiments give us no means of estimating the actual values of $\frac{\alpha_{Cl_P}}{\alpha_{Cl_E}}$ or $\frac{\alpha_{B_E}}{\alpha_{B_P}}$; but in somewhat similar experiments Hastings, Salvesen, Sendroy, and Van Slyke found $\frac{[B_E]}{[B_P]}$ about equal to $\frac{\alpha_{H_E}}{\alpha_{H_P}}$.

Now

$$\frac{\alpha_{H_E}}{\alpha_{H_P}} = \frac{\alpha_{B_E}}{\alpha_{B_P}} = \frac{f_{B_E}\,[B_E^+]}{f_{B_P}\,[B_P^+]}$$

so that

$$\frac{[B_E^+]}{[B_P]} = \frac{f_{B_E}\,[B_E^+]}{f_{B_P}\,[B_P^+]} \qquad \text{......(15)},$$

from which it appears probable that

$$f_{B_E} = f_{B_P} \qquad \text{......(16)},$$

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[1] The sum of the errors in these estimations should not exceed 2 %; the actual difference found between $[Cl_P]\,[B_P]$ and $[Cl_E^-]\,[B_E^-]$ always exceeds 3 %.

and that $[\mathrm{B}_P] = [\mathrm{B}_P^+]$, in other words that all the kation is ionised (if unionised kation were present f_{B_P} would have to be actually greater than f_{B_E}, which is improbable).

From (13), (14) and (15) we infer that $f_{\mathrm{Cl}_P} < f_{\mathrm{Cl}_E}$, or that $[\mathrm{Cl}_P] > [\mathrm{Cl}_P^-]$ (that is, that some of the Cl present in the protein solution is in an undissociated condition even on the alkaline side of the iso-electric point). Since $\frac{[\mathrm{Cl}_P]\,[\mathrm{B}_P]}{[\mathrm{Cl}_E^-]\,[\mathrm{B}_P^+]}$ decreases, if anything, with increasing p_{H}, it is probable that the first is the correct explanation, that is, that $f_{\mathrm{Cl}_P} < f_{\mathrm{Cl}_E}$.

The lowest value of $\frac{f_{\mathrm{Cl}_P}}{f_{\mathrm{Cl}_E}}$ so reckoned is 0·935, so that the effect of protein on the activity coefficient is not great; it appears to increase only slightly when the p_{H} increases from about 6·7 to about 8·0, that is when the charge on the protein is about doubled. Although the protein ion is polyvalent, the charges are probably so far apart that they have little more effect on the activity coefficients of other ions than would equivalent charges on separate ions; it is quite possible that weak electric fields round numerous polar groups at the links between individual amino-acids have more effect than do the few charges on the molecule.

The excess of diffusible ions found in the protein solution is much greater than calculated by equation (9) on the assumption that the activity coefficients of the diffusible ions were unaffected by the protein. It is not possible to reckon the osmotic pressure due to this difference simply with the aid of the calculated activity coefficients, for the osmotic coefficient involves an integration and requires a knowledge of the equation connecting activity coefficient and protein concentration; but the actual osmotic pressure due to this difference is not necessarily less than that calculated for ideal solutions.

Observed osmotic pressures.

It can be seen from Fig. 3 and Table II that the osmotic pressures found for the same serum, at the same hydrogen ion concentration and salt concentration in the external fluid, agree very well; the differences between the three sera used (two human and one horse) are what would be expected from the difference of the albumin to globulin ratios. The only result which is out of agreement is that of Exp. 4 *a*, which was obtained by the first method and the discrepancy in the case is not too great to be accounted for by the disturbances that occurred in this experiment.

Our results agree well with those of Verney [1926] and Mayrs [1926]; the osmotic pressures are uniformly higher owing to the higher temperature at which we have worked.

An increase of osmotic pressure with a rise of the p_{H} was detected in our experiments in which the more accurate method was used, even with low protein concentrations (Exps. 13 *c* and *d*), and with such small changes of p_{H}

Table II. *Osmotic pressures of serum-proteins.*

Experiment Later method	Serum	p_H ext. fluid	[Pr]	$\frac{\text{Albn.}}{\text{Globn.}}$	Osmotic pressure cm. of water 0°	37°	Remarks
9 *a*	L. H.	6·7	67·6	$\frac{2\cdot9}{1}$	29·9	32·8	
9 *b*	,,	6·7	40·9	$\frac{2\cdot9}{1}$	15·4	17·6	
11 *a*	,,	6·4	74·0	$\frac{2\cdot8}{1}$	33·6	36·0	
12 *a*	,,	7·5	68·6	$\frac{2\cdot8}{1}$	33·2	36·8	
12 *b*	,,	7·8	68·6	$\frac{2\cdot8}{1}$	—	37·5	
10 *a*	,,	7·8	63·6	$\frac{2\cdot9}{1}$	30·1	33·65	
10 *b*	,,	7·8	36·4	$\frac{2\cdot9}{1}$	13·5	15·9	
8 *a*	J. M.	6·4	64·8	$\frac{2\cdot3}{1}$	27·7	29·9	
8 *b*	,,	6·4	21·2	$\frac{2\cdot3}{1}$	—	7·4	
11 *b*	,,	6·4	57·2	$\frac{2\cdot2}{1}$	—	24·9	
12 *c*	,,	7·5	50·3	$\frac{2\cdot2}{1}$	—	22·9	
12 *d*	,,	7·8	50·3	$\frac{2\cdot2}{1}$	—	23·3	
13 *a*	,,	6·8	62·3	$\frac{2\cdot2}{1}$	27·8	29·9	
13 *b*	,,	8·0	62·3	$\frac{2\cdot2}{1}$	—	32·2	
13 *c*	,,	6·8	38·6	$\frac{2\cdot2}{1}$	15·0	15·6	
13 *d*	,,	8·0	38·6	$\frac{2\cdot2}{1}$	—	16·4	
Earlier method							
3 *a*	,,	6·9	50·4	—	—	22·3	
5 *a*	,,	6·7	63·9	$\frac{2\cdot37}{1\cdot00}$	—	30·3	
5 *b*	,,	6·7	26·3	$\frac{2\cdot37}{1\cdot00}$	—	11·1	
4 *a*	,,	8·3	67·8	$\frac{2\cdot40}{1\cdot00}$	—	33·3	Temperature regulation disturbed
4 *b*	,,	8·3	37·1	$\frac{2\cdot40}{1\cdot00}$	—	15·8	Temperature regulation disturbed
6 *a*	,,	6·4	66·9	$\frac{2\cdot31}{1\cdot00}$	—	33·3	Ext. fluid about 0·3 *M*
6 *b*	,,	6·4	36·7	$\frac{2\cdot31}{1\cdot00}$	—	17·7	,, ,, ,,
7 *a*	,,	8·3	64·4	$\frac{2\cdot31}{1\cdot00}$	—	32·0	,, ,, ,,
7 *b*	,,	8·3	32·3	$\frac{2\cdot31}{1\cdot00}$	—	13·5	,, ,, ,,
1 *a*	Horse	6·9	70·5	$\frac{0\cdot88}{1\cdot00}$	—	29·6	
1 *b*	,,	6·9	42·5	$\frac{0\cdot88}{1\cdot00}$	—	15·9	
2 *a*	,,	8·2	68·9	$\frac{0\cdot88}{1\cdot00}$	—	29·7	
2 *b*	,,	8·2	42·6	$\frac{0\cdot88}{1\cdot00}$	—	15·7	

as that from 7·5 to 7·8 (Exps. 12 *a*, *b*, *c*, *d*). The changes of osmotic pressure found were, however, much less than those calculated for ideal solutions by equation (9). It is probable that the increase of total osmotic pressure due to the greater excess of diffusible ions in the protein solution at the higher p_H is to some extent balanced by a reduction of the activity coefficient of the protein molecules when they are more highly ionised; such a reduction of activity coefficient with increased ionisation has been found by Cohn and Prentiss [1927] in the case of haemoglobin.

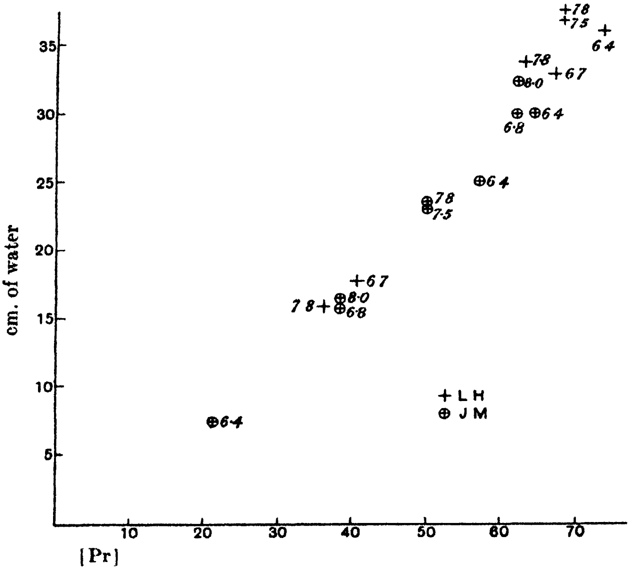

Fig. 3.

It is not therefore necessary to assume from the slight effect of change of p_H that the Donnan osmotic effect is much less than that calculated for ideal solutions and inadequate to account for a large part of the deviation of the protein concentration-osmotic pressure curve from a straight line.

With increase of the salt concentration of the external fluid the Donnan effect should be proportionately reduced, and consequently the relation between protein concentration and osmotic pressure should be more nearly linear. This we have found in Exps. 6 *a*, *b* and 7 *a*, *b*.

The changes of osmotic pressure with temperature are on the whole rather less than would be expected. The irregularity in the results is probably due to inaccuracies in the estimation at 0°. as the time allowed at this temperature was probably insufficient for the full pressure to be registered in all cases; if this is the case the lower values for $\frac{\text{O.P. at } 37^\circ}{\text{O.P. at } 0^\circ}$ are probably the more correct. The results at any rate suggest that there is no great change in the physical condition of the proteins in passing from 0° to 37°.

Hydration of protein.

The relation of osmotic pressure to protein concentration can be explained as shown by Verney by supposing that the protein in solution occupies a much greater volume than it occupies when dry; this could only be the case if the protein was surrounded by a large water sheath ("hydration") or enclosed by a large volume of water (as probably occurs in gelatin solution). The latter supposition can be shown (from consideration of the osmotic pressures found and the possible area of the protein molecule) to be impossible in the case of the proteins we are considering. The solubility of non-polar substances, such as nitrogen [Stoddart, 1927], in protein solutions shows that hydration of proteins, in the sense that lithium ions, for example, are hydrated, does not occur to any great extent. It may be supposed that proteins, in some unknown way, hold a sheath of water which behaves as a solvent just as does the main bulk of the water, the whole mass forming an "omnibus" of protein, water and other solutes. It must however be remembered that the chief evidence for this so-called "hydration" is based on the high viscosities of protein solutions which can be explained in other ways.

We are now further studying osmotic pressures and the effect of proteins on activity coefficients, using individual proteins.

SUMMARY.

1. The observed osmotic pressures of serum-proteins rise with increase of p_H.

2. This increase is less than that calculated for ideal solutions if the compound of protein and base is fully dissociated.

3. The activity coefficients of anions are slightly reduced by the presence of proteins in solution.

4. Both the excess of diffusible ions in the protein solution (due to the Donnan effect) and deviations from ideality will cause the observed osmotic pressures of proteins not to bear a linear relation to the protein concentration. It is not necessary to suppose that proteins in solution occupy a greater bulk than they do when dry.

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CLIV. EXPERIMENTS ON NUTRITION. VIII.

COMPARATIVE VITAMIN B VALUE OF FOODSTUFFS. CEREALS. I.

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It became evident from our previous experiments [Plimmer, Rosedale and Raymond, 1927] that all the constituents of the diet needed balancing by vitamin B to ensure satisfactory rearing of chickens. A similar balance was found in the cases of pigeons and rats. On a basal diet free from vitamin B, large quantities, such as 4 % of dried yeast, or 8 % of marmite, were found necessary to maintain pigeons for long periods.

It is essential to know not only the constituents of the diet which supply vitamin B, but also and more particularly the relative amount of it contained in each foodstuff. Cooper [1912, 1914] and Chick and Hume [1917] have given a most valuable series of comparative data, and there are a few figures by American workers The figures of Cooper and of Chick and Hume have been expressed in terms of daily quantities on a fixed daily intake of food. From these data figures bearing a relation to the food consumption can be calculated. No quantitative information is available about the common cereals, like whole wheat, rye, maize, though data were given for wheat germ. bran, barley. There is also little quantitative information about vegetables, fruits and other foodstuffs.

These experiments have therefore been undertaken to obtain these facts about the commoner foodstuffs and thus add to our knowledge about food analysis. Some cereals and cereal products have so far been tested and experiments are in progress with other foodstuffs. The figures are purely of relative value. The exact amount of vitamin B in foodstuffs cannot be known till the substance has been isolated and identified, and even then it may not be possible for some time to devise a method for its direct estimation in foodstuffs. Meanwhile feeding experiments are required for information about nutrition.

EXPERIMENTAL METHODS.

The vitamin B value of foodstuffs was tested by Cooper and by Chick and Hume by methods referred to as the curative and the preventive. The former gives information more rapidly and is most useful for testing the value of extracts. The results depend to a considerable extent upon the actual state of the pigeon affected by polyneuritis. Sometimes the birds rapidly reach the condition with retracted head and are easily cured; sometimes this condition is reached more slowly and the cure is more difficult. These peculiarities have also been observed by Kinnersley and Peters [1925]. The preventive method gives more exact information about a foodstuff, as its effect is observed over a considerable period, usually from 60 to 100 days. The pigeons were fed artificially with 30 to 40 g. of white rice per day and a daily quantity of the foodstuff under examination. On account of the laboriousness of the method only a few foods were tested. The results of the two methods have agreed fairly well, but there were some considerable divergences.

Our previous observations [Plimmer and Rosedale, 1923] on two pairs of pigeons on a diet of oatmeal and fishmeal have led us to adopt the preventive method. These birds were maintained for a long period and were allowed to feed *ad lib.* The food consumption amounted to 40 to 45 g. per day. They were not able to rear their young without the addition of marmite to the food. In these experiments instead of artificial feeding we have fed the birds *ad lib.* and we have taken as our standard long maintenance with rearing of young. This standard is much more severe and is possibly too high. At any rate a period of 100 days is sometimes too short. We have often observed maintenance for this time and then the birds have lost their appetite with consequent loss of weight and have died of polyneuritis. Loss of weight always accompanies loss of appetite and at present it is not yet proved that another factor than the antineuritic is concerned in their nutrition.

Foodstuffs can be placed in several groups according to the amount of vitamin B that they contain. Those with none, like white rice and white flour, those very rich, like dried yeast and wheat germ, those with intermediate quantities, which may be much or little.

Our method of testing has been to take a foodstuff, such as whole wheat, to add to it 5 % of fishmeal to supply protein and mineral salts, and see if a pair of birds could be maintained on it with reproduction. If this occurred, then a proportion of white rice (10 %) was added and reproduction once more awaited. Further dilutions of the grain or other food were then made with more white rice, until it was found that the pigeons could no longer rear their young. It was not necessary to add an antiscorbutic to the food, nor has it been found necessary to add cod-liver oil to supply fat-soluble vitamins.

This standard of testing has been found rather too severe, as in some cases there has been maintenance in perfect health without reproduction. It has been observed that if the food is satisfactory the birds eat the rice first; if not satisfactory, the rice is left. White flour has then been substituted for white rice. This has the advantage that the food mixture can be made into a dough with water and the dough can be made into pills and dried. The birds must then either eat the whole food or leave it. The minimum quantity for maintenance on such a mixture has still to be found in several cases. The results of the experiments are given in a series of tables and additional information is supplied by a short further description.

All the birds have been kept in cages as described previously [1927] on the roof of the building, conditions which are more natural than small cages for one bird or for several. The birds were kept as quiet as possible so as to prevent neglect of their eggs and young ones.

OATMEAL.

The two pairs of adult pigeons, which had already been kept for over a year on oatmeal and fishmeal, were continued on the same diet, with or without the addition of marmite, until the end of 1923. The red pair (Nos. 1 and 2) hatched and reared three pairs of young with marmite; a fourth pair, started with marmite, died on removal of the marmite. The blue pair (Nos. 4 and 5) without marmite had five lots of eggs and failed to rear the young. On including marmite in the food, a pair of young was reared. The details about these pairs of birds are given in Table I.

Table I. *Oatmeal.*

		Diet				Weights, g.			
Date	Time in weeks	Oatmeal	Marmite	Cod-liver oil	Fishmeal	Cock No. 1	Hen No. 2	Number of eggs	Result
14. vi. 23		90	1·5	0	5	510	500	6 during June, July, Aug.	4 hatched and reared; 1 cracked; 1 unfertile
to								2 in Sept.	Both hatched; 1 squab died on 27th day, no cause of death found. Marmite now omitted and 2nd squab lost weight and died
2. xi. 23	20								
3. xi. 23		90	0	0	5	500	545		
to									
31. xii. 23	9					555	655		
						No. 5	No. 4		
14. vi. 23		90	0	1	5	395	390	10 during June, July, Aug., Sept.	All fertile; 7 died at hatching; 3 hatched and died
to									
17. x. 23	18								
18. x. 23		90	1·5	1	5	445	420	2 in Oct.	Both hatched and reared
to									
31. xii. 23	11					505	430		

The blue pair (Nos. 4 and 5) were continued on the oatmeal diet for the next two years with the results shown briefly in Table II. This pair was used mainly as a control for all experiments with the different cereals.

At first, 1·5 % marmite was added and a pair of young was hatched and reared for a week. The hen then unfortunately escaped and the young were deserted by the cock. With a new hen, after a single egg from which the squab died, two young were reared. Marmite was then raised to 2 %, but the eggs laid were spoilt.

Table II. *Oatmeal.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Oatmeal	Marmite	Fishmeal	Cock No. 5	Hen No. 4		
1. i. 24		93·5	1·5	5	480	390	4 in Jan., Feb.	1 broken; 1 unfertile; 2 hatched. Hen escaped; squabs died
to								
18. iii. 24	11							
						No. 6		New hen
19. iii. 24		93·5	1·5	5	460	390	3 in May, June	1 hatched and died; 2 hatched and reared
to								
27. v. 24	10							
28. v. 24		93	2	5	425	445	2 in July	Fertile and deserted
to								
23. vii. 24	8							
24. vii. 24		95	0	5	420	450	2 in Aug.	Both hatched; 1 died; 1 reared
to							2 in Feb.	1 broken; 1 died at hatching
29. iv. 25	40				420	375		
30. iv. 25							1 in April	Hen died; egg fertile and deserted. Cock only
to								
24. vi. 25	8				400			
						No. 4		New hen
25. vi. 25		95	0	5	400	380	12 during June, Aug., Sept., Oct., Nov.	3 unfertile; 3 hatched and reared, but poor growth and squabs unwell; 2 deserted; 2 dead in shell; 2 hatched and died
to								
17. xii. 25	25				540	510		

It was desired to test oatmeal alone over a long period. In 48 weeks only two lots of eggs were laid, from which only one squab was reared. After 6 months two more eggs were laid but young were not reared. A single egg was then laid and deserted. The hen was then unwell and died. The cock was also losing weight at this period. With a new hen (No. 4) one young was reared from the first two eggs, both were reared from the next eggs, and subsequently no more were reared.

Adult pigeons can thus be maintained for long periods on a diet of oatmeal and young can occasionally be reared. Young can generally be reared with an addition of marmite (2 %). The long period on oatmeal caused a slight loss of weight of the adult birds, the hen being more affected than the cock, possibly from loss of vitamin B from her body into the eggs. One hen died on the long period of oatmeal feeding.

Oatmeal would thus appear to contain just enough vitamin B for maintenance of pigeons, but not enough for rearing.

SUSSEX GROUND OATS.

The red pair of pigeons (Nos. 1 and 2) was changed over to Sussex ground oats on 1. i. 1924. The hen laid two lots of eggs in the first period of 20 weeks, but no young were reared. In the next two periods, amounting together to 21 weeks, marmite was added. Young ones were again not reared. The cause of non-rearing seemed due rather to the fibrous nature of the food than to lack of vitamin B. The young thrived for a time and died with fibre in the gizzard. At the end of the breeding season the adult birds were kept a final 11 weeks on Sussex ground oats and both increased in weight.

Sussex ground oats are thus similar to oatmeal. Adult pigeons can be maintained, but young cannot be reared.

The details are shown briefly in Table III.

Table III. *Sussex ground oats.*

		Diet			Weights, g.			
Date	Time in weeks	Sussex ground oats	Marmite	Fish-meal	Cock No. 1	Hen No. 2	Number of eggs	Result
1. i. 24 to 20. v. 24	20	95	0	5	495	600	4 in Mar., Apr.	3 died after hatching; 1 fertile, not hatched
21. v. 24 to 18. vii. 24	9	91	4	5	435	490	4 in May, June	1 unfertile; 3 hatched and died
19. vii. 24 to 13. x. 24	12	89	6	5	465	510	4 in July, Aug.	2 addled; 2 hatched and died in 1st week
14. x. 24 to 31. xii. 24	11	95	0	5	440 430	495 510		

WHOLE OATS.

The pair was changed over to whole oats for a period of one year (1925). The change had a favourable effect upon the rearing of young. Both young from the first pair of eggs were reared, one from the second lot, and one from the fourth lot. The first young ones were practically normal, but the later ones showed poor growth and were not first-class birds on removal from their parents. The adult pair maintained their healthy appearance throughout, but increased in weight too much at the end. The difference in result between Sussex ground oats and whole oats is probably due to fibre. The husk of the whole oat was usually discarded by the birds, whilst such separation was not possible from the ground oats.

Whole oats appear rather better than oatmeal. In the preparation of oatmeal, a portion of the germ with vitamin B seems to be removed.

The details are shown in Table IV.

Table IV. *Whole oats.*

		Diet			Weights, g.			
Date	Time in weeks	Whole oats	Marmite	Fish-meal	Cock No. 1	Hen No. 2	Number of eggs	Result
1. i. 25		95	0	5	430	510	8 in Mar., May, June, July	4 hatched and reared, poor growth never quite normal; 2 addled; 2 hatched and died in 2nd week
to								
30. xii. 25	52				535	655		

Barley.

This pair of birds (Nos. 21 and 22) was given barleymeal for the first year (Table V). The fertility of the eggs was not good and changes in the diet had to be made after the rearing of a single squab. No squabs were produced with the diet of 70 % barleymeal. On this quantity the pair was kept for 20 weeks: no loss in weight of the birds occurred.

Table V. *Barley.*

		Diet			Weights, g.			
Date	Time in weeks	Barley-meal	Rice	Fish-meal	Cock No. 21	Hen No. 22	Number of eggs	Result
1. i. 24		90	0	5	420	460	6 in Mar., Apr., May	2 unfertile; 2 hatched and reared; 1 fertile, deserted; 1 hatched and died
to								
1. vii. 24	25							
2. vii. 24		80	10	5	395	415	2 in June, July	1 unfertile; 1 hatched and reared
to								
12. viii. 24	7							
		Barley grains						
13. viii. 24		70	20	5	420	495	4 in Aug., Sept.	2 addled; 2 unfertile
to								
31. xii. 24	20							
1. i. 25		70	20	5	420	500	2 in Jan.	Deserted, unfertile. Cock lost
to								
10. ii. 25	7				No. 121			New cock
to					410	480		
13. iv. 25	9						2 in Feb.	2 hatched and reared
14. iv. 25		60	30	5	410	470	8 in Apr., May, July, Aug.	1 unfertile; 1 addled; 6 hatched and reared
to								
4. v. 25	3	63·5	31·5	5				
to								
8. vi. 25	5	65	30	5				
to								
5. x. 25	18							
6. x. 25		55	40	5	420	465	6 in Jan., Feb., Mar.	4 broken and deserted; 1 died at hatching; 1 died in 2nd week with symptoms of polyneuritis. Hen died, blood clot near heart
to								
26. iii. 26	24				380	480		
					No. 25/34			New hen
2. iv. 26					390	400	6 in May, June, Aug.	3 hatched and reared; 1 dead in shell; 2 fertile, and deserted
to								
20. i. 27	42				480	500		

During the second year barley grains were substituted for barleymeal. The birds had experienced some difficulty in picking up the fine meal and consequently complete consumption of the food was not really attained. With grains of barley, it was observed that all the food was picked up and eaten.

Unfortunately the cock escaped soon after this change. With a new cock, the fertility of the eggs was improved and a pair of young was reared on 70 % barley.

On reducing the barley to 60 %, then raising to 63·5 % and later to 65 %, squabs were hatched and reared.

Reduction of the barley to 55 % did not allow of rearing. One squab which was hatched died with symptoms of polyneuritis. Subsequently the hen died. With a new hen, altogether three squabs were reared—the next eggs were fertile and deserted and then no more eggs were laid. This condition of maintenance without reproduction has been observed in other cases.

55 % of barley thus seems the limiting quantity for rearing, but 65 % is better. It will be necessary to ascertain the limiting quantity for maintenance in future experiments.

RYE.

Whole rye seeds were used as food for these birds (Nos. 9 and 10). As shown in Table VI, the birds were first given rye and fishmeal. After the rearing of

Table VI. *Rye.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Rye	Rice	Fish-meal	Cock No. 10	Hen No. 9		
5. iii. 24		95	0	5	375	415	2 in Apr.	Both hatched and reared
to								
27. v. 24	12				395	420		
28. v. 24		80	10	5			2 in May	Both hatched and reared
to								
8. vii. 24	6				390	420		
9. vii. 24		70	20	5			12 in July, Aug., Sept., Oct., Jan., Mar.	10 fertile, deserted; 2 hatched and reared
to								
22. iv. 25	41				430	435		
23. iv. 25		60	30	5			2 in May	Both hatched and reared
to								
10. vi. 25	7	63·5	31·5	5	390	415		
11. vi. 25		55	40	5			4 in June, July	1 addled; 1 died at hatching; 2 hatched and reared
to								
9. ix. 25	13				450	425		
10. ix. 25		45	50	5			2 in Feb.	Both hatched and reared
to								
14. iv. 26	31				400	425		
15. iv. 26		40	55	5			2 in Apr.	1 fertile, deserted; 1 hatched and reared. Birds do not eat rice
to								
7. vii. 26	12				400	405		
8. vii. 26		45	50	5				Birds still leave rice
to								
22. ix. 26	11				345	370		
23. ix. 26		50	45	5				Rice eaten; birds better
to								
24. xi. 26	9				360	395		

a pair of squabs, rice was added in gradually increasing quantities according as young were reared. The birds were kept for the long period of 41 weeks with 70 parts of rye, mainly on account of the eggs being deserted. This was largely due to the very wet season; the nest of the birds was continually swamped with water. On reducing the rye to 60 parts, young were hatched from the first pair of eggs. Young were hatched and reared with as little as 45 % of rye. With less rye in the diet, the adult birds after a time did not eat the rice and began to look unwell. The quantity was obviously too little. No great improvement in the birds was seen on raising the rye to 45 %, but on giving 50 % rye the birds improved. It can be concluded that adult birds require 45 % rye for maintenance and that 50 to 55 % is required for rearing of young. It will be of interest in future experiments to prove definitely whether a minimum of 40 or 45 % is needed for maintenance, which will correspond with the figure found for wheat.

WHEAT.

Whole wheatmeal flour, such as is used for human consumption, was given as the food for these birds (Nos. 17 and 18) (see Table VII). At first, the flour was wetted with water to bind the fine particles together, but later, on mixing with rice, the two components (the rice finely ground) and the fishmeal were made into a dough, and the dough was made into small pills which were dried on glass plates at room temperature or at 37°. Two young were reared up to a proportion of 70 % wheat. Many eggs were laid on the diet with 60 % wheat during the long period of 31 weeks, but no young were hatched. The proportion of whole wheat was then raised first to 70 % and then to 75 %. Again young were not hatched out. The hen then died. No particular symptoms could be found at *post mortem* examination and it seemed that she died of exhaustion from laying so many eggs, 30 in all.

With a new hen, keeping the whole wheat at 75 %, during the long period of 51 weeks, only two young were hatched and reared. Two other pairs of young were hatched, but they died in the second week with symptoms of polyneuritis. The other eggs were fertile, but no young were hatched from them.

It thus appeared that 75 % whole wheat was the minimum for the hatching and rearing of young, but that 80 % was distinctly better.

The quantity of whole wheat in the diet was now reduced to 60 %. The eggs which were laid were fertile, but always deserted.

In the meantime another pair of birds had been kept on 50 % whole wheatmeal (see below) and it was decided to find out what was the minimum quantity of whole wheatmeal required for the maintenance of adult birds. The quantity of whole wheatmeal was therefore reduced to 30 %. On this quantity both birds died, which showed definitely that more than 30 % was needed.

Another pair of birds (Nos. 25/2 and 25/3) was kept on 50 % whole wheatmeal, which was mixed with white flour instead of rice, for 45 weeks. One young was hatched and reared from the first pair of eggs. The other eggs were fertile and were deserted. To test whether an increase of whole wheatmeal flour to 75 % would improve the hatching and rearing, the birds were given this diet for another 34 weeks. Rather more eggs were laid, but they were always deserted. During the time on 50 % whole wheat the birds kept a constant weight. This quantity thus seemed a satisfactory amount for long maintenance.

Table VII. *Wheat.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Wheat	Rice	Fish-meal	Cock No. 17	Hen No. 18		
26. iii. 24 to 28. v. 24	10	90	0	5	440	365	4 in Apr., May	2 hatched and reared; 2 deserted
29. v. 24 to 23. vii. 24	8	80	10	5	430	370	2 in June	Both hatched and reared
24. vii. 24 to 3. ix. 24	6	70	20	5	425	365	4 in July, Aug.	2 hatched and reared; 2 deserted
4. ix. 24 to 7. iv. 25	31	60	30	5	440	365	14 in Sept., Oct., Nov., Jan., Feb., Mar., Apr.	4 unfertile; 10 addled and deserted
8. iv. 25 to 6. v. 25	4	70	20	5	450	385	2 in May	1 unfertile; 1 fertile, deserted
7. v. 25 to 17. vi. 25	6	75	20	5	445	380	4 in May, June	1 very small egg; 2 unfertile; 1 addled. Hen died; no particular symptoms
						No. 118		New hen
1. vii. 25 to 26. vi. 26	51	75	20	5	400 430	335 365	12 in July, Oct., Feb., Mar., May	1 addled: 2 hatched and reared; 2 hatched and died in 2nd week with enlarged hearts; 4 deserted; 2 dead in shell; 1 hatched, died in 2nd week with polyneuritis
27. vi. 26 to 29. ix. 26	14	60	35	5	450	415	7 in June, July, Aug., Sept.	5 deserted, fertile; 2 dead at hatch
30. ix. 26 to 7. iii. 27	23	30	65	5	350		2 in Oct.	Deserted, fertile. Hen died on 31. xii. 26 after 13 weeks. Cock died of polyneuritis
					No. 25/2	No. 25/3		
18. xi. 25 to 29. ix. 26	45	50	45	5	440 415	445 465	8 in Feb., Apr., June, July	1 dead in shell; 1 hatched and reared; 1 hatched and died; 5 fertile and deserted
30. ix. 26 to 25. v. 27	34	75	20	5	435	460	9 in Oct., Feb., Mar., Apr., May	8 fertile and deserted 1 unfertile
					No. 93	No. 26/27		
10. iv. 27 to 17. viii. 27	23	40	55	5	455 385	380 295	6 in Apr., May, June	1 hatched and reared, but very poor growth; 2 unfertile; 3 fertile and deserted

It remained to ascertain if 40 % whole wheatmeal would be enough for maintenance. Another pair of birds (Nos. 93 and 26/27) was started on this quantity. They managed to rear one young one from the first lot of eggs, but this young one showed very poor growth and was very small. Two other lots of eggs were laid which were deserted and then no more. They have been kept for over 20 weeks without signs of illness, but they have shown a slight loss in weight.

It may be concluded that 40 % of whole wheat is the minimum for maintenance of adult pigeons, 50 % is a more favourable quantity and 75 to 80 % whole wheat is required for reproduction and rearing of young.

WHEAT GERM.

The high vitamin B value of wheat germ was determined by Chick and Hume [1917]. They found that pigeons on a daily diet of 30 to 40 g. of white rice were protected over a period of 63 days by a dose of 3 g. of wheat germ given every second day. This amount corresponds to about 4·5 % of the diet. According to our experiments the smallest quantity which must be present in the diet is 6 %. Table VIII shows our results. The first period was for

Table VIII. *Wheat germ.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		White rice	Wheat germ	Fish-meal	Cock No. 24/13	Hen No. 24/10		
23. vii. 24 to 11. xi. 24	16	90	10	5	360	330		
12. xi. 24 to 17. ii. 25	14	90	6	5	445	395		Birds found to be two hens
		White flour			No. 31	No. 24/10		
18. ii. 25 to 30. vi. 25	19	89	6	5	460	440	8 in Feb., Mar., Apr., May, June	1 unfertile; 2 addled; 2 dead at hatching; 3 fertile and deserted
1. vii. 25 to 20. xi. 25	20	87	8	5	425	385	6 in July, Aug.	All deserted. 24. viii. 25, hen ill, dosed with 4 doses of 2 g. wheat germ; better. Hen died with polyneuritis; enlarged heart
						No. 25/31		New hen
25. xi. 25 to 1. vi. 26	27	85	10	5	460	460	4 in Mar., Apr.	Fertile and deserted
2. vi. 26 to 27. vii. 26	8	93	2	5	430	395	1 in June	Fertile, deserted. Hen died 7. vii. 26, in 5 weeks. Cock died
					No. 26/2	No. 26/1		
8. vii. 26 to 22. ix. 26	11	91	4	5	345	395	2 in July	Deserted. Both birds died of polyneuritis

16 weeks with a proportion of 10 % of wheat germ and was followed by a period of 14 weeks with 6 %. The supposed cock of this pair of birds was then discovered to be a hen. On exchanging one of the hens for a cock the pair was kept for 19 weeks on 6 % of wheat germ. At this time white flour was used instead of white rice, and the diet given in small pills.

The hen laid eggs during this period, but they were always deserted. The quantity of wheat germ was then raised to 8 % to see if any improvement in hatching would take place. The hen showed signs of polyneuritis after 8 weeks, but was cured with four doses of 2 g. of wheat germ. At 20 weeks the hen again suffered from polyneuritis and died.

Another hen was placed with the cock and the amount of wheat germ was raised to 10 %. The hatching was no better and after 27 weeks the attempt to produce rearing was discontinued.

It was decided to find out the smallest quantity of wheat germ which would suffice to maintain a pair of pigeons. The quantity was therefore reduced to 2 %. Both birds died in 8 weeks.

Table IX. *Heated wheat germ.*

		Diet			Weights, g.			
Date	Time in weeks	White rice	Heated wheat germ	Fish-meal	Cock No. 24/12	Hen No. 24/14	Number of eggs	Result
23. vii. 24		90	10	5	315	370		Cock lost
to					No. 9131			New bird
11. xi. 24	16				430	440		
12. xi. 24		90	6	5	365	430		New bird found to be hen
to								
11. ii. 25	13				450	450		
to					No. 25			Another cock
					380	410		Hen died from unknown cause
4. iii. 25	3				390	395		
		White flour				No. 24/23		New hen
11. iii. 25		89	6	5	375	370	6 in Apr., May, June	4 fertile and deserted; 1 addled; 1 died at hatching
to								
30. vi. 25	16							
1. vii. 25		87	8	5	405	395	10 in July, Aug., Sept., Oct.	2 deserted; 1 unfertile; 5 hatched and died; 2 addled
to								
18. xi. 25	20				430	430		
19. xi. 25		85	10	5			14 in Nov., Dec., Jan., Feb., Mar., Apr.	5 unfertile; 3 fertile and deserted; 5 broken; 1 addled
to								
12. v. 26	25				390	400		
13. v. 26		83	12	5			2 in May, June	1 egg only at each time of laying; both broken
to								
28. vii. 26	10							Cock escaped
					No. 26/6	No. 26/3		
27. vii. 26		91	4	5	405	385		Cock died in 6th week; hen died in 8th week
to								
22. ix. 26	8							

Another pair was started on 4 % wheat germ. These died in 11 weeks. 2 % and 4 % wheat germ were therefore insufficient to balance the white flour. On 6 % two birds were kept for 14 weeks and another pair for 19 weeks. One of the two had been for both these periods on this quantity. 6 % must therefore be taken as the minimum quantity for long maintenance.

HEATED WHEAT GERM.

At the same time as the experiments with wheat germ were being made, similar ones with heated wheat germ were carried out. The wheat germ is heated to about 120° to prevent its decomposition. Table IX shows the results. A pair of birds was kept for 16 weeks with 6 % heated wheat germ. There was a slight improvement in hatching with 8 and 10 % wheat germ.

A pair of birds on 4 % of heated wheat germ died in 8 weeks. For maintenance therefore 6 % of wheat germ is required. The germ does not lose vitamin B by this treatment[1].

BRAN AND MIDDLINGS.

The high content of vitamin B in bran was found by Chick and Hume [1917]. It appeared to be about one-fifth of that of wheat germ.

Table X. *Bran.*

		Diet				Weights, g.			
Date	Time in weeks	Bran	White flour	Fish-meal	Marmite	Cock No. 24/4	Hen No. 24/6	Number of eggs	Result
9. vii. 24 to 2. ix. 24	8	45	45	5	0	365	365		
3. ix. 24 to 14. iv. 25	32	30	60	5	0	395	455	6 in Jan., Feb., Mar.	4 died at hatching; 1 addled; 1 fertile, deserted
15. iv. 25 to 22. ix. 25	23	47·5	47·5	5	0	430	425	10 in Apr., May, June, July, Aug., Sept.	3 died at hatching; 2 unfertile; 2 addled; 3 hatched and died
23. ix. 25 to 2. ii. 26	19	33	60	5	2	420	450	4 in Oct., Jan.	2 hatched, 1 died 1 reared; 2 fertile and deserted
3. ii. 26 to 18. v. 26	15	33·3	57·7	5	4	430	455	4 in Feb., Apr.	2 unfertile, deserted; 2 addled, deserted
19. v. 26 to 22. vi. 26	5	33·3	55·7	5	6	385	410	2 in May	Deserted
					Dried yeast				
23. vi. 26 to 29. ix. 26	14	33·3	55·7	5	6	380 415	400 425	5 in July, Sept.	1 egg only and deserted; 4 fertile and deserted

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[1] The wheat germ and heated wheat germ were kindly supplied by Mr W. T. Ryle. Messrs Hovis, Ltd., have also kindly given us wheat germ for these experiments.

Table XI. *Middlings.*

		Diet				Weights, g.			
Date	Time in weeks	Middlings	White rice	Fish-meal	Marmite	Cock No. 24/3	Hen No. 24/5	Number of eggs	Result
9. vii. 24 to 2. ix. 24	8	70	20	5	0	370	335		
3. ix. 24 to 9. xii. 24	14	60	30	5	0	365	355		
10. xii. 24 to 31. iii. 25	16	30	60	5	0	415	385	4 in Feb., Mar.	1 unfertile; 1 addled; 2 dead at hatching
			White flour						
1. iv. 25 to 16. vi. 25	11	30	60	5	0·6	435	380	5 in Apr., May, June	1 deserted; 2 addled; 1 unfertile; 1 died at hatching
17. vi. 25 to 13. i. 26	30	33·3	60	5	1·7	405	375	10 in June, July, Aug., Sept., Nov.	4 deserted; 2 hatched and reared; 4 hatched and died
14. i. 26 to 5. v. 26	16	33·3	58·7	5	3·0	495	440	8 in Jan., Feb., Mar., Apr.	2 dead at hatch; 6 broken, deserted
6. v. 26 to 23. vi. 26	7	33·3	55·7	5	6·0	435	375	4 in May, June	Broken and deserted
					Dried yeast				
24. vi. 26 to 29. ix. 26	14				6·0	395 430	335 360	2 in July	Broken. Hen ill 30. viii. 26

In order to obtain further comparative figures for these foodstuffs pairs of pigeons were started on 45 % bran and on 70 % middlings. As shown in Tables X and XI, the pair was kept on 30 % bran for 32 weeks. As they were not able to rear young the amount of bran was raised to 47·5 %. Some eggs were hatched, but the young died. On account of the large amount of fibre in bran, extra vitamin B was supplied by incorporating a proportion of marmite; one young squab was then reared. With a further increase of marmite, no more young were reared and there was no improvement on changing from marmite to dried yeast. The attempt to rear was then abandoned.

Similar results were obtained with middlings. There was better hatching and rearing on adding marmite to the diet. With still more marmite, there was no better rearing. It would appear that some other factor than vitamin B was now concerned in reproduction. Both with bran and middlings the weights of the parent birds remained practically constant.

As far as maintenance of adult pigeons is concerned, the pair was kept on 30 % bran for 32 weeks, and the pair on 30 % middlings for 16 weeks. In future experiments it is proposed to ascertain the smallest quantities of bran and middlings upon which adult pigeons can be maintained for 20 to 26 weeks.

MAIZE.

Kibbled maize (see Table XII) was used as food for these pigeons (Nos. 13 and 14). At first, partly owing to the wet weather, the eggs were deserted. An addition of marmite was made to the food and ultimately a squab was reared.

Table XII. *Maize.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Kibbled maize	Marmite	Fishmeal	Cock No. 14	Hen No. 13		
19. iv. 24 to 27. vi. 24	10	90	0	5	445	385	6 in Apr., May, June	4 fertile and deserted; 2 addled
28. vi. 24 to 8. viii. 24	9	90	2	5	455	400	2 in July	Addled
9. viii. 24 to 24. x. 24	11	90	3	5	465	420	2 in Sept.	1 addled; 1 hatched and reared
25. x. 24 to 29. v. 25	31	90	0	5	500	440	14 in Oct., Nov., Dec., Jan., Mar., Apr.	4 spoilt by wet weather; 4 fertile and deserted; 1 addled; 5 hatched and reared
			White rice					
30. v. 25 to 4. ix. 25	14	85	5	5	435	395	8 in June, July, Aug.	4 hatched and reared; 2 deserted; 2 hatched and died
5. ix. 25 to 30. ix. 26	56	75	20	5	470	415	23 in Oct., Nov., Jan., Feb., Mar., Apr., May, June, July, Aug.	6 hatched and reared; 6 unfertile; 4 addled; 7 fertile and deserted
1. x. 26 to 9. iii. 27	23	50	45	5	450	410	16 in Oct., Nov., Dec., Jan., Feb.	6 fertile and deserted; 10 broken
10. iii. 27 to 1. vi. 27	9	95	0	5	495 435	425 395	7 in Mar., Apr., May	All broken
					No. 26/4	No. 26/5		
29. vii. 26 to 16. iii. 27	33	60	35	5	410 455	355 410	6 in Jan., Feb., Mar.	3 fertile and deserted; 1 hatched and died; 2 dead at hatch
			White flour					
17. iii. 27 to 20. vii. 27	18	60	35	5	435	390	7 in Apr., May, June, July	5 fertile and deserted; 1 fertile and dead at hatch; 1 hatched and died

On returning to the simple maize and fishmeal diet, several young were hatched and reared. The maize thus appeared to contain enough vitamin B for rearing. After adding white rice to the diet, young were hatched and reared. The pair was kept on 75 % kibbled maize for the long period of 56 weeks during which time 6 squabs were reared. This proportion of maize thus seemed the limit for rearing. No young were reared on 50 % maize, though the parents were kept on it for 23 weeks. On returning to kibbled maize only, rearing was not improved. After the long period of over two years the experiment with these birds was not continued. Lack of the power of rearing was probably due to exhaustion of some other factor which is required.

Another pair of pigeons was in the meantime tried on 60 % kibbled maize. Though many eggs were laid, and some hatched out, there was no rearing of the young.

75 % maize must be regarded as the minimum for rearing. So far 50 % has been found enough for maintenance, but 60 % was distinctly better. Future experiments are designed to find out if pigeons can be maintained on less.

Maize Germ-meal and Maize Gluten.

These two cereal products are often used in feeding poultry and other animals.

The high vitamin B value of maize germ, picked by hand from the grain, has been shown by Chick and Hume [1917]. It was consequently expected that maize germmeal would be rich in vitamin B.

A pair of pigeons was started on 90 parts rice, 10 parts maize germ-meal and 5 parts fishmeal. They lost weight at once and looked ill. On raising the maize germ-meal to 20 parts, there was no improvement and the birds died in altogether 11 weeks.

On trying a pair of pigeons on 90 parts maize germ-meal and 5 parts of fishmeal, they became ill and died in 6 weeks.

Another pair was tried with 90 parts maize germ-meal, 90 parts white flour and 5 parts fishmeal. They also became ill, but recovered on adding 5 % of marmite to the mixture. On reducing the marmite to 3 parts, the quantity to balance the white flour, they became ill and died.

Commercial maize germ-meal thus appears to contain no, or very little, vitamin B.

Maize gluten was not expected to contain vitamin B. The following experiments showed the presence of a small amount. A pair of birds was given 45 parts maize gluten, 45 parts white flour, 5 parts fishmeal and 5 parts marmite. They showed growth for 6 weeks, but on reducing the marmite to 3 parts they lost weight and died.

A pair of birds on 90 parts maize gluten and 5 parts fishmeal died in 4 and 14 weeks.

Maize gluten may thus contain small amounts of vitamin B.

Buckwheat.

Buckwheat, often used in America for human consumption and in France for feeding poultry, has been tested on the pair of pigeons Nos. 23 and 24

(Table XIII). After 18 weeks on buckwheat alone, a pair of squabs was reared. One young one was reared on 80 parts of buckwheat. The hen subsequently laid no more eggs. The pair was maintained without loss of weight for 31 weeks. The cock then died. With a new cock there was no improvement, even after returning to buckwheat alone as the diet. Finally a small proportion of cod-liver oil was added; eggs were still not laid by the hen. This grain seems to differ from the other grains by not permitting the laying of the eggs by the hen.

Further experiments are needed to ascertain the peculiarities of buckwheat.

Table XIII. *Buckwheat.*

Date	Time in weeks	Diet			Weights, g.		Number of eggs	Result
		Buck-wheat	White rice	Fish-meal	Cock No. 23	Hen No. 24		
20. ii. 24 to 25. vi. 24	18	90	0	5	420	285	7 in Mar., Apr., May	1 little egg; 2 shell-less; 1 hatched and died; 3 hatched and reared
26. vi. 24 to 5. viii. 24	6	80	10	5	415	300	2 in June	1 unfertile; 1 hatched and reared
6. viii. 24 to 11. iii. 25	31	70	20	5	450 395	330 340	No eggs	Cock died; tumour
12. iii. 25 to 16. ix. 25	35	95	0	5	365 400	340 335	No eggs	New cock
17. ix. 25 to 6. xii. 25	12	94·5	0	5	435	345	No eggs	With 0·5 % cod-liver oil

MILLET AND DARI.

These two grains much used as human food by the natives of South Africa with good results showed a high vitamin B value on testing on pigeons. Tables XIV and XV show the behaviour of the pigeons. On gradually reducing the proportion of millet in the diet, very good hatching and rearing took place with 60 parts of millet. With 50 parts of millet the rearing was less good, but was slightly improved with 55 parts. The minimum for rearing can be said to be 55 %. Smaller quantities to determine the figure for maintenance have not yet been tried. The birds have been quite well on 50 % for 18 weeks.

The pair of birds on dari were quite prolific, but the fertility of their eggs was not good. A pair of squabs was reared on 50 %. The quantity for rearing is more certain at 60 %.

The maintenance quantity has not been determined, but experiments for this purpose will be undertaken shortly. The pair of birds lived quite well on 50 % dari.

Table XIV. *Millet.*

		Diet			Weights, g.			
Date	Time in weeks	Millet	White rice	Fish-meal	Cock No. 15	Hen No. 16	Number of eggs	Result
1. v. 24 to 25. vi. 24	8	90	0	5	350	380	2 in May	Both hatched and reared
26. vi. 24 to 26. viii. 24	9	80	10	5	375	365	4 in June, July	2 addled; 2 hatched and reared
27. viii. 24 to 22. x. 24	8	70	20	5	415	395	2 in Aug., Sept.	1 dead in shell; 1 hatched and reared
23. x. 24 to 31. xii. 24	10	60	30	5	430	400	2 in Nov.	Both hatched and reared
1. i. 25 to 5. v. 25	18	50	40	5	480 420	425 415	6 in Jan., Mar., Apr.	1 dead in shell; 1 unfertile; 2 hatched and reared; 2 hatched and died
6. v. 25 to 9. vi. 25	5	53	42	5			2 in May	1 dead in shell; 1 hatched and died
10. vi. 25 to 12. x. 25	18	55	40	5	395	380	6 in June, July, Sept.	4 dead in shell; 1 hatched and reared; 1 unfertile
13. x. 25 to 23. xi. 25	6	60	35	5	435	430	2 in Oct.	Both hatched and reared
24. xi. 25 to 25. i. 26	9	55	40	5	440 495	435 430	2 in Dec.	Both hatched and reared

BAKER'S YEAST.

Our experience with dried yeast and marmite led to testing the value of baker's yeast. Analysis of a sample showed 2·23 % of N (= 13·9 % of protein), 1·9 % of ash and 71·3 % of water, which would indicate a value of about one-third of dried yeast.

An experiment was started with 10 % of baker's yeast. The birds at first increased slightly in weight, then remained constant for about 20 weeks. During this time the hen laid three lots of eggs. They were fertile, but were deserted. Subsequently, both birds lost their appetite and their weight and died at the end of the 26th week with the typical retracted head symptoms of polyneuritis. Table XVI gives the further information.

Another experiment was made with 4 % of baker's yeast, the amount corresponding with the necessary amount of dried yeast. The birds did not like the food and scattered it. They lost weight at once and both showed polyneuritis in the fifth week. The cock was cured by a dose of 2 g. of wheat germ, but the hen was allowed to die.

Table XV. *Dari.*

		Diet			Weights, g.			
Date	Time in weeks	Dari	White rice	Fish-meal	Cock No. 7	Hen No. 8	Number of eggs	Result
20. ii. 24 to 23. vi. 24	18	90	0	5	400	420	7 in Mar., Apr., May, June	2 unfertile; 2 fertile and deserted; 2 deserted; 1 hatched and reared
24. vi. 24 to 8. viii. 24	8	80	10	5	475	380	2 in July	Both hatched and reared
19. viii. 24 to 6. x. 24	7	70	20	5	490	395	2 in Aug.	1 died at hatching; 1 hatched and reared
7. x. 24 to 17. xi. 24	6	60	30	5	490	435	2 in Oct.	1 died at hatching; 1 hatched and reared
18. xi. 24 to 6. iv. 25	20	50	40	5	570	440	6 in Dec., Jan., Feb.	3 unfertile; 1 fertile and deserted; 2 hatched and reared
7. iv. 25 to 8. vi. 25	9	45	45	5	520	415	4 in Mar., May	3 hatched and reared; 1 unfertile
9. vi. 25 to 30. vi. 25	3	50	45	5	450	400	2 in June	Both died at hatching
1. vii. 25 to 13. x. 25	15	55	40	5	440	380	6 in July, Aug., Sept.	1 hatched and reared; 1 unfertile; 2 addled; 2 dead at hatching
14. x. 25 to 24. xi. 25	6	60	35	5	500	440	2 in Oct.	Both hatched and reared
25. xi. 25 to 5. i. 26	6	55	40	5	530 590	410 450	2 in Dec.	Both unfertile

Table XVI. *Baker's yeast.*

		Diet			Weights, g.			
Date	Time in weeks	White flour	Baker's yeast	Fish-meal	Cock No. 53	Hen No. 48	Number of eggs	Result
16. ii. 27 to 17. viii. 27	26	85	10	5	415 440 400 410 340	320 365 320 335 220	6 in Apr., May, June	All fertile and deserted. Both birds died of polyneuritis

Discussion and Summary.

At the time that these experiments were started, it was generally understood that the term vitamin B applied to the antineuritic substance preventing beri-beri. Yeast and other foods have sometimes been described as possibly containing two unknown food factors, the one antineuritic and the other maintaining body weight and necessary for growth. The work of Goldberger and his colleagues, and, more recently, that of Chick and Roscoe [1927] again tend to differentiate two factors in yeast and wheat embryo. The one factor is definitely antineuritic and can be destroyed by heating at 120° under pressure; the other is thermostable, preventive of pellagra and maintains body weight and is called by Goldberger the PP factor. Chick and Roscoe wish to call this PP factor vitamin B. The nomenclature of the various unknowns in nutrition is already sufficiently complicated, *e.g.* two D vitamins have been described; the one water-soluble in yeast (Funk) and the other fat-soluble, now more definitely called vitamin D. Funk's vitamin D may be the same as the PP factor. It would have been simpler to leave the term vitamin B for the antineuritic substance with which it has so long been associated and give the term PP factor to the unknown substance in yeast and other foods which prevents pellagra and maintains body weight.

The term antineuritic limits the symptoms to those of paralysis, but a deficiency of this factor is responsible for a whole sequence of other symptoms relating to the alimentary tract, the endocrine organs and the heart, as shown by McCarrison, and observed by us on feeding with too little yeast [1927]. The animal grows, showing the presence of the second factor, but dies with alimentary, heart, or paralytic symptoms according to the degree of shortage of the vitamin.

The existence of two forms of beri-beri, the wet and the dry, has long been known, and it has often been suggested that they were due to two factors. Vedder was never able to produce the dropsical condition in birds, even with extra salts in the diet. Our observations again point to the different forms as being due to the degree of shortage of vitamin B. We have often seen wet tissues in our birds (more often in chickens) when the degree of shortage is slight and exists over a long period. A great shortage causes an early death and the tissues are dry. Before death the bird has generally stoppage of the cloaca. If excretion should occur, the birds get better for a time. If a bird is cured with a dose of yeast or wheat germ, there is first of all excretion and then recovery.

With reference to the unknown factor concerned in preventing pellagra, it is not definitely proved that it is not a constituent of proteins, as Goldberger originally believed. The experimental work of Chick and Roscoe was done with caseinogen, a protein which cannot be looked upon as a complete one. Caseinogen is deficient in cystine and may also lack other protein units. These deficiencies are probably compensated for by lactalbumin in milk, which has been shown to be so efficacious in preventing and curing pellagra and also promotes growth. Yeast protein may contain the same unit and similarly wheat embryo, especially the less pure samples which are better for pellagra prevention and for maintaining body weight. It would be of great interest to know if the same results are obtained with lactalbumin, or other complete protein. The protein in our experiments has been fishmeal.

In any case we hope that Chick and Roscoe will consider our plea to continue the association of the term vitamin B with the antineuritic factor and adopt the term PP factor for the unknown factor in yeast concerned in pellagra prevention and in maintaining body weight. McCollum and Davis associated the antineuritic substance with vitamin B more particularly from the fact that impure lactose contained vitamin B and would cure polyneuritis in pigeons, rather than from the similar distribution of the substances.

Our results are considered entirely in respect of the antineuritic substance. We have observed previously [1927] that young birds and rats need more of it than adult animals. These data with cereals support those observations. Adult pigeons can be maintained on a smaller quantity of cereal than that upon which young can be reared. The same argument can be applied to the PP factor, that young animals want more than adults, but against this contention is the fact that the young bird grows quite rapidly and then dies showing symptoms of polyneuritis, so that it appears to have enough PP factor. Our observations have also always been to the effect that the bird, whether young or adult, first loses its appetite with corresponding loss of weight and then dies of polyneuritis. The quantity of antineuritic factor is the determining consideration.

Our data may be summarised very simply as follows:

	Percentage quantity of foodstuff required for	
	Rearing	Maintenance
Oatmeal	More than 95	95
Sussex ground oats ...	More than 95	95
Whole oats	95	95
Barley (whole) ...	65	55
Rye (whole)	50–55	45
Whole wheat flour ...	75–80	40–50
Wheat germ	More than 10	6
Bran	—	33
Middlings	—	33
Maize (kibbled) ...	75	50–60
Maize germ-meal ...	Contain very little vitamin B	
Maize gluten		
Buckwheat	80	70
Millet	55	50
Dari	60	50
Dried yeast	—	4
Baker's yeast	—	10–12
Marmite	—	8–10

As pointed out previously, some of the quantities for maintenance may be less than shown in this table. It is curious to see that the common cereal grains, wheat, rye, barley, have about the same value, that millet and dari have a rather greater value, maize a little less, and that oatmeal is least good in respect of vitamin B.

In general, our figures for maintenace are higher than those calculated from the data of Chick and Hume and of Cooper. The explanation is that our experiments were for a longer duration than 100 days. We have frequently seen polyneuritis on an amount corresponding to that of Chick and Hume after 15 weeks and occasionally after 20 or even 25 weeks. The minimum quantity adopted by us is that which keeps the birds in health for 26 or more weeks.

The relative value of the different cereals is shown better by adopting one of the above foods as a standard. It is most convenient to adopt the food richest in vitamin B, namely dried yeast, and give it the figure of 100. Then, by putting any of the above amounts for maintenance equal to 4 (dried yeast), the relative amount is given by calculating for 100 of the cereal.

Thus: 40 whole wheat = 4 dried yeast, therefore 100 whole wheat = 10; 50 whole wheat = 4 dried yeast, therefore 100 whole wheat = 8; 6 wheat germ = 4 dried yeast, therefore 100 wheat germ = 66.

The series of relative values is then as follows:

Relative vitamin B values of foods.

Dried yeast	100	Rye	9	Bran	12–13
Baker's yeast	33–40	Barley	7–8	Middlings	12–13
Marmite	40–50	Oats	4–5	Maize	7–8
Wheat germ	66	Oatmeal	4	Millet	8
Whole wheat	8–10	Buckwheat	5–6	Dari	8

It is further not difficult to calculate from these relative figures the quantity of food that must be contained in a diet. By dividing 400 by the above figures one obtains the percentage amount that should be included in a diet otherwise devoid of vitamin B. Another way of determining the amount of cereal, or a mixture of cereals, that should be present in a diet is by including the quantity, or quantities, that supply enough to give a total of 4. Thus, 20 % of millet will give 1·6, 10 % of dari will give 0·8, 40 % of whole wheat will give 3·2. Total = 5·6. The remainder of the diet can be white rice, or white flour, or fishmeal or any foodstuff without vitamin B.

These figures are for pigeons. For chickens, the amount must be calculated on a basis of 6, instead of 4. For rats, the vitamin B requirement is less, about half that of the pigeon, so that its amount will be calculated on a total of 2. The chief question is the requirement of man. He can be considered like a rat with the figure of 2, but it would be safer to consider that his requirement approaches that of a pigeon. The only figure that we can deduce for man is based on the experience in the Japanese Navy. Beri-beri was prevented by a ration of barley amounting to approximately one-third of the dry weight of the total food. This quantity, if it be assumed to be a minimum, gives a figure of not more than 3 for man's requirement, between that of a rat and a pigeon.

We acknowledge with thanks a grant from the Government Grant Committee of the Royal Society which has in part defrayed the cost of these experiments.

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CLV. THE ESTIMATION OF CYANOGENETIC GLUCOSIDES.

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(*Received August 30th, 1927.*)

As a preliminary to a study of the behaviour of the cyanogenetic glucoside (prulaurasin) in the leaves of cherry laurel (*Prunus laurocerasus*) an attempt was made, at the suggestion of Dr F. F. Blackman, F.R.S., to find a method for the estimation of the glucoside in a single leaf or half leaf. After a trial of various methods the most satisfactory was found to be by decomposition of most of the glucoside by heating the leaf in water and by decomposition of the remainder by added emulsin. During both processes a current of air was passed through the generating vessel into potash. The HCN liberated during the decomposition and absorbed in the potash was titrated in the alkaline solution with silver nitrate (Liebig's method).

Apparatus.

This was composed of six units each connected by equal lengths of fine bore capillary tubing (to equalise the bubbling rates) to a bottle exhausted by a good filter pump. The six generating vessels, 500 cc. filter flasks, were placed in a water-bath which could be heated to boiling and cooled by changing the water.

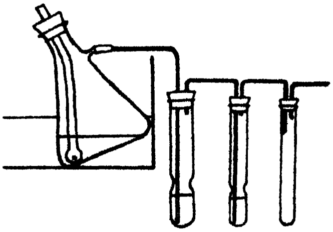

Fig. 1.

Each generating flask (Fig. 1) had an inlet tube through the stopper with fine openings below the surface of about 100 cc. of water in the bottom of the tilted flask. The side tube of the flask was connected to two test-tubes, in series, with their inlet tubes reaching to the bottom and perforated there with fine holes. The first test-tube was 3 cm. in diameter and contained 15 cc. of 5 % potash, and the second 1·5 cm. in diameter and contained 5 cc. of potash. Each of these test-tubes had a constriction just above the level of the liquid contained in it, which served as a means of checking frothing and promoting circulation. The outlet tube of the second test-tube was connected to a third with short inlet and outlet tubes. This served to catch any potash which might have frothed over. The last test-tube was connected to the equalising resistance tubing. The contact with rubber tubing was made as small as possible.

Method.

The air current is turned on so as to produce violent bubbling and the first of the weighed leaves is cut up into small narrow pieces as quickly as possible, on a sheet of glazed cardboard, by means of a sharp scalpel. The pieces are then rapidly pushed into the first flask. The other five leaves are treated in a similar manner and put into their respective flasks and the temperature of the bath is raised to 100° during a quarter to half an hour and kept at this temperature for several minutes. The heating is then stopped and the temperature of the bath lowered by running in cold water and running out the hot. About 0·05 g. of emulsin is then added to each flask together with a few drops of amyl alcohol to check frothing.

The time during which the air current should be continued varies with the air rate and other peculiarities of the apparatus and is best determined from a curve of the rate of exhaustion of HCN from amounts of amygdalin similar to those of the glucoside to be estimated. With the apparatus described 12 hours appeared sufficient, so that in practice 14–18 hours' run was given.

Titration of the HCN. The contents of the second and third test-tubes are washed into the first. The resulting volume of liquid (about 25 cc.) is approximately halved by the constriction in the tube. Advantage is taken of this to obtain rapid titration. The tube is placed in a box with three sides and the floor blackened inside and the fourth side open and facing a source of bright diffused light. A standard amount (about five drops) of 5 % potassium iodide solution is added and $M/100$ silver nitrate is run in from a 10 cc. microburette. The silver nitrate is mixed, by means of a stirring rod flattened at the end, with the upper half of the solution only, and this can be titrated quickly to a slight turbidity. A further amount of silver nitrate almost equal to the first is then run in and the whole mixed. By repeating this process of titrating the upper half and then mixing the whole solution, the end-point (a very slight turbidity) can be reached rapidly without fear of over-titrating. The blackened box ensures that the first trace of turbidity will be recognised by means of the Tyndall effect. The potassium iodide must be added since the amounts of HCN are here too small for the precipitation of AgCN to act as an indicator. In the presence of KI the turbidity is due to the precipitation of AgI immediately there is excess of silver nitrate over that required to form the double compound AgCN.KCN.

1 cc. of $M/100$ silver nitrate = 0·0005404 g. of HCN or 0·00590 g. of prulaurasin.

DISCUSSION OF VARIOUS METHODS.

Estimation of the glucoside by means of the benzaldehyde liberated was rejected owing to the liability of this substance to oxidation [Armstrong and Horton, 1908].

Estimation of the glucoside sugars by the amount of change in optical rotation or copper-reducing power after the addition of enzyme involves the extraction of the glucoside before estimation. Complete extraction is difficult and it will be shown later that dropping the leaves into boiling absolute alcohol, to kill them before the estimation, involves a loss of about 6 % of the glucoside, judged by the liberation of HCN which takes place at this stage.

Distillation method.

Evidence has been produced by several workers [Alsberg and Black, 1916; Willaman, 1917] that the distillation of plant tissue with acid results in incomplete recovery of the HCN from the cyanogenetic glucosides.

This was confirmed by comparison with the aeration method, using cherry laurel leaves. Direct distillation with acid gave a recovery of 27 % and distillation after maceration 14 %. Distillation without acid gave 49 % and subsequent distillation with acid a further 3 %. These recoveries are calculated by taking as 100 % the HCN content, determined by the aeration method, of the corresponding halves of the leaves.

It can be seen from this that the distillation method is not a reliable quantitative method. Distillation without acid gives higher yields than with it.

The distillation of small amounts of amygdalin with acid gave similar incomplete yields (20–30 % recoveries with 10 % acid).

When KCN solution was distilled with acid there was a 95–97 % recovery of the HCN. When benzaldehyde was added as well only 50–70 % was recovered.

The apparent reaction between benzaldehyde and HCN at the boiling point may explain the similar losses which occur during the distillation of the prulaurasin of cherry laurel and of amygdalin.

Aeration method.

The other method investigated was based on that of Roe [1924] for the estimation of amygdalin, where the glucoside is decomposed by means of emulsin and the HCN carried by a rapid current of air from the generating liquid into potash.

The method was tested with small amounts of amygdalin.

With 0·0400 g. of amygdalin in each experiment Recoveries	With 0·00348 g. of amygdalin in each experiment Recoveries
98·9 %	99·4 %
102 %	99·6 %
100·2 %	96·9 %
100·0 %	98·2 %

In applying the aeration method to leaves, steam was first used to inactivate the glucoside-hydrolysing enzyme, but it was found that nearly all the glucoside had been decomposed, as shown by the HCN liberated, before the inactivation occurred. Treub [1907] also found that he could not prevent the decomposition of less than ½ to ¾ of the glucoside when suddenly heating leaves in steam.

The volatile product, HCN, here gives evidence of a great deal of enzyme action before the enzyme is inactivated. It suggests that in other cases, where the enzyme is supposedly inactivated instantaneously by plunging into boiling water or by steaming, a great deal of decomposition may occur before the inactivation takes place.

Plunging the leaf into boiling absolute alcohol was then tried, and even with this a part of the HCN is liberated (about 6 %). The cut leaves were dropped into the boiling alcohol in a flask, through which a current of air was passed into potash. The alcohol was distilled over into the potash, water and a small amount of emulsin were then added to the leaves, and the liberated HCN carried over into the potash in an air current. It was found that the alcohol had only a very slight effect on the end-point of the titration. This loss of HCN when leaves are dropped into boiling alcohol has also been shown by Treub [1907].

This "alcohol" method, although complicated, ensures that the action of other enzymes shall not interfere. The liberation of HCN at high temperatures and at the time of opening the flask is avoided. Hence the results were regarded as probably showing the whole of the HCN present as glucoside in the leaf.

After the alcohol method had been elaborated a simpler one for class experiments was devised in collaboration with the Hon. Mrs Onslow. In this the leaves were placed in cold water and gradually heated to 100° in a current of air. After cooling to 35° emulsin was added and the aeration carried on so that traces of glucoside remaining were decomposed.

Most of the glucoside is hydrolysed by the enzyme of the leaf while the temperature is being raised to 100°, but some still remains and is split by the added emulsin.

	(1)	(2)
HCN given off at the end of heating	95·5 %	91·3 %
HCN given off after emulsin addition	4·5 %	8·7 %

Owing to the slower heating the enzyme is inactivated at a temperature of 60–80° and the apparent loss of HCN which takes place in the distillation method does not take place here; since the results by this "water" method agree with those by the "alcohol" method, where nearly all (about 94 %) of the decomposition is by the added emulsin at 35°. The agreement between the two methods is shown by the following experiments with the corresponding halves of leaves.

In one experiment the results were 100 : 100·4 (HCN by "water" method: HCN by "alcohol" method), and in another 100 : 104·6. Since this was near the limit of experimental error and within the variations between the corresponding halves of leaves, this was taken as showing that the simpler water method was about as accurate as the "alcohol" method. Hence the arguments on the non-interference of enzymes, etc. in the alcohol method must hold for the "water" method also. The simpler method was adopted in the final apparatus.

Estimation of the HCN. Since in the aeration method it is necessary to absorb the HCN in potash, titration in the alkaline solution by silver nitrate was the most convenient method. When the necessary precautions were taken this was found to be quite accurate enough for the present biological requirements. Results by this method were found to agree with those by the iodine method [Dunstan and Henry, 1904].

Using the blackened box and small volumes of titrated fluid the addition of one drop from the microburette (0·01 cc.) is sufficient to give a distinct end-point in clear solutions. Hence readings may be obtained accurate to 0·02 cc. of $M/100$ $AgNO_3$, which corresponds to 0·00001 g. of HCN. Thus the quantities of HCN in a single leaf, 0·001–0·005 g., can be estimated with sufficient accuracy.

It is difficult to obtain an idea of the error of the whole process when used with leaves, but with amygdalin in amounts corresponding to those of prulaurasin in a cherry laurel leaf, the error is 1–3 %. The HCN contents per 100 g. of fresh weight of the corresponding halves of leaves also agree within these limits.

It is of course open to question whether the HCN as measured in this way is representative of the cyanogenetic glucoside in the plant. This however seems probable, since in the "alcohol" method it is only the β-glucosidic HCN which can be liberated, as nearly all the decomposition is by added emulsin. Further, by the kindness of Mrs Onslow, I am able to state that the average of some of her unpublished determinations of the glucoside sugar of young cherry laurel leaves is in substantial agreement with the aeration results for corresponding leaves; moreover, data obtained during starvation by the two methods fall on closely parallel curves. The values for glucoside sugar give a somewhat lower amount of equivalent HCN. This is what would be expected since a certain amount of decomposition must have taken place, previous to the estimation, during the killing of the leaves in boiling absolute alcohol.

SUMMARY.

Various methods of estimating the cyanogenetic glucosides in plant tissues are discussed and most of them considered unsatisfactory. In particular the distillation method has been found to give untrustworthy results.

Two new methods are described in which the HCN of the glucoside is liberated by enzyme action and carried over into potash in a current of air. The HCN absorbed is titrated in the alkaline liquid by silver nitrate. This method is considered satisfactory if certain details of procedure are adopted.

The "alcohol" method is considered to be a sound method for the estimation of cyanogenetic glucosides in leaves.

The results by this method agree with those by a simpler "water" method which was adopted for use in plant physiological investigation. The details of apparatus and procedure used in this are described.

It is hoped to publish elsewhere data obtained by this method on cherry laurel leaves.

This research was carried out during the tenure of a grant from the Department of Scientific and Industrial Research and later of the Frank Smart Studentship in Botany. I should like to tender my very grateful thanks to Dr F. F. Blackman, F.R.S., and to the Hon. Mrs Onslow for help and advice throughout the investigation.

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CLVI. NEUTRAL SALT ADDITION COMPOUNDS OF ALKALINE EARTH GLUTAMATES AND ASPARTATES.

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(*Received September 1st, 1927.*)

It was shown a few years ago by King and Palmer [1920] that glycine could form a series of addition compounds with alkali halides and alkaline earth halides under experimental conditions free from dubiety, thus extending and confirming the earlier work of Pfeiffer and his co-workers. About the same time Hugounenq and Florence [1920], by the action of calcium carbonate upon glutamic acid hydrochloride, obtained an addition compound which they represented by either of the formulae

$$C_4H_8O_2N.CO_2CaCl.H_2O \text{ or } (C_4H_8O_2N.CO_2)_2Ca.CaCl_2.2H_2O,$$

but they were unable to isolate analogous compounds using barium or other metallic oxides. A year later [1921] the same observers prepared from the reaction product of aspartic acid hydrochloride and chalk, by alcoholic precipitation, a calcium chloride addition compound analogous to that from glutamic acid.

As no further work has appeared on these interesting products, which are essentially glycine derivatives with the added complication of an extra carboxyl group in salt formation, the subject has been examined more closely in the hope of deciding between the formulae advanced by Hugounenq and Florence and of arriving at some conclusion as to the mode of attachment of these inorganic addenda.

d-Glutamic acid was chosen as starting material since it is optically active and can be readily obtained in large quantities. In the experimental portion a process is described whereby 30·5 g. of pure *d*-glutamic acid hydrochloride may be obtained from 100 g. of gluten flour avoiding alcohol as a precipitant and also the laborious process of saturation with hydrogen chloride gas. In addition an effective modification of Hugounenq and Florence's method for preparing glutamic acid from the hydrochloride is described.

When glutamic acid hydrochloride, hydrobromide or hydriodide is boiled in aqueous solution with freshly precipitated calcium or barium carbonate or with the corresponding hydroxide followed by saturation with carbon dioxide, the filtrates, on concentration, readily deposit well-formed crystals of the new addition compound of the type

$$(CO_2H.CHNH_2.CH_2.CH_2.CO_2)_2\,M.MA_2.xH_2O,$$

where M may be calcium or barium and A may be chlorine, bromine, or iodine.

In the case of strontium, the first crystalline crop was invariably strontium glutamate, so for purposes of comparison calcium and barium glutamates were prepared from free glutamic acid and found to be much more soluble than strontium glutamate. The exceptional behaviour of the strontium solutions is thus due to the solubility product of strontium glutamate being reached before that of the addition compounds. In confirmation of this it was found that if solutions of strontium glutamate were allowed to crystallise in presence of excess of strontium chloride, bromide or iodide, then addition compounds of the same type as those formed with calcium and barium halides were readily obtained.

In a similar manner aspartic acid hydrochloride and strontium carbonate gave the addition compound

$$(CO_2H.CHNH_2.CH_2.CO_2)_2\,Sr.SrCl_2.2H_2O$$

without the need for excess of strontium chloride, and definite evidence was also obtained for the formation of an addition compound between barium chloride and barium aspartate, but only in the presence of large excess of barium chloride. The calcium analogue obtained by Hugounenq and Florence by precipitation with alcohol could not be obtained by us, using aqueous media alone.

The chief interest of these compounds apart from their possible physiological significance lies in their constitution. The starting materials are optically active and a determination of the variation of specific rotation with concentration over a wide range of concentrations might be expected to cast some light upon their constitution. The results of such an experiment are shown graphically in Fig. 1, where a number of addition compounds are compared with the corresponding alkaline earth glutamates and alkali glutamates. The general trend of the curves is similar, from a region of positive rotation at high concentrations to a region of negative rotation at low concentrations, the whole corresponding to a change of molecular rotating species with dilution. At high dilutions, 1–2 %, the curves show a tendency to flatten out, and it is of interest that if the ionic value for the negative glutamate ion be calculated from these curves at a concentration in the region of 2·5 % then the ionic values show a measure of agreement.

Salt	$[\alpha]_{5461}$ for ion	Salt	$[\alpha]_{5461}$ for ion
$(C_5H_8O_4N)_2\,Ca.H_2O$	−6·3°	$(C_5H_8O_4N)_2\,Ca.CaCl_2.2H_2O$	−5·8°
$(C_5H_8O_4N)_2\,Ba.5\frac{1}{4}H_2O$	−5 8°	$(C_5H_8O_4N)_2\,Ca.CaBr_2.2H_2O$	−5 8°
$(C_5H_8O_4N)_2\,Sr.4H_2O$	−6·1°	$(C_5H_8O_4N)_2\,Ba.BaCl_2.6H_2O$	−6·3°
$C_5H_8O_4N$ Li	−5·9°	$(C_5H_8O_4N)_2\,Ba.BaBr_2$	−6·2°
$C_5H_8O_4N$ Na	−5·5°	Mean	−6·0°

It seems justifiable to conclude that at high concentrations comparable indeed with saturated solutions, the non-ionised alkaline earth glutamates and the non-dissociated additive compounds with alkaline earth halides are dextro-rotatory and possibly that the addition compounds do not markedly alter the constitution of the rotating unit.

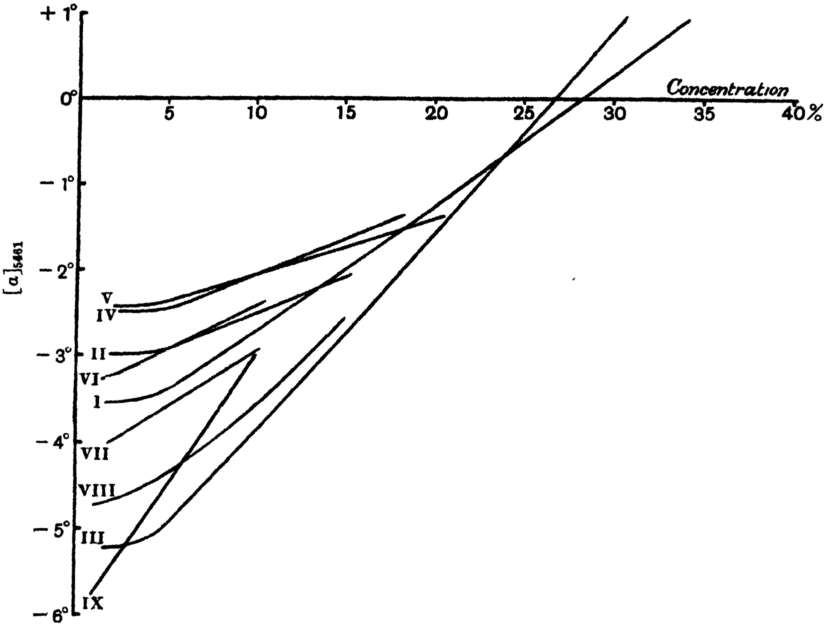


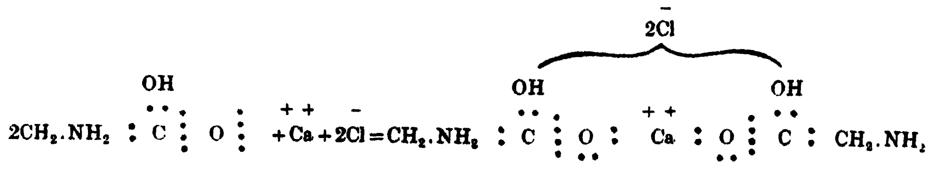
Fig. 1.

I = $(C_5H_8O_4N)_2Ca.CaCl_2.2H_2O$
II = $(C_5H_8O_4N)_2Ca.CaBr_2.2H_2O$
III = $(C_5H_8O_4N)_2Ca.H_2O$
IV = $(C_5H_8O_4N)_2Ba.BaCl_2.6H_2O$
V = $(C_5H_8O_4N)_2Ba.BaBr_2$
VI = $(C_5H_8O_4N)_2Ba.5\frac{1}{4}H_2O$
VII = $(C_5H_8O_4N)_2Sr.4H_2O$
VIII = $C_5H_8O_4N\,Na$
IX = $C_5H_8O_4N\,Li$

The constitution of the addition compounds of glycine and neutral salts has been ably discussed by Pfeiffer [1922] in terms of Werner's theory of residual affinity and co-ordination. There has however, as far as we are aware, been no discussion of these particular complexes from the point of view of electron sharing. The most frequent type of compound formed by glycine is

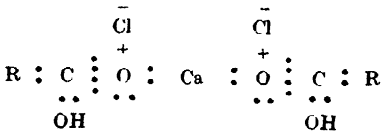
$$2CH_2NH_2.CO_2H.MA_2.xH_2O$$

where M is an alkaline earth and A is a halogen. This is readily interpreted electronically as follows, the indication of the electrons being here confined to atoms actually participating in the reaction



$$2CH_2.NH_2 : \overset{OH}{C} : O : + \overset{++}{Ca} + 2\overset{-}{Cl} = CH_2.NH_2 : \overset{\overbrace{\text{OH} \qquad\qquad \text{OH}}^{2\overset{-}{Cl}}}{C : O : \overset{++}{Ca} : O : C} : CH_2.NH_2$$

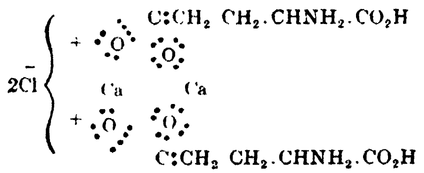
In the latter formula two electrons from each carbonyl oxygen atom enter the outer shell of the calcium atom, the oxygen atoms which were originally neutral thereby becoming positively charged and the calcium atom becoming doubly negatively charged. The calcium atom was however initially doubly positively charged through ionisation of the chlorine ions, and the resultant neutralisation of charges on the calcium atom results in the chlorine ions being held to oxygen by oxonium valencies, thus



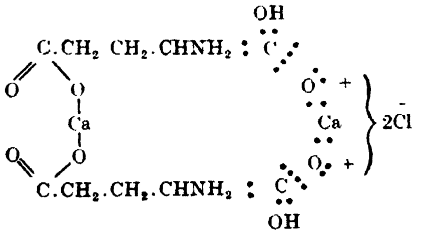
$$R : \underset{OH}{C} : \overset{\overset{-}{Cl}}{\overset{+}{O}} : Ca : \overset{\overset{-}{Cl}}{\overset{+}{O}} : \underset{OH}{C} : R$$

In these compounds therefore calcium has a co-ordination number of two.

The alkaline earth glutamate and the aspartate addition compounds described in this paper are all of one type and fall into the same general scheme of electron sharing. Owing however to the presence of two carbonyl oxygen atoms in each glutamic or aspartic acid molecule there are two possible formulae and there is no evidence to decide between these. Thus the addition compound of calcium chloride and calcium glutamate can be written either as



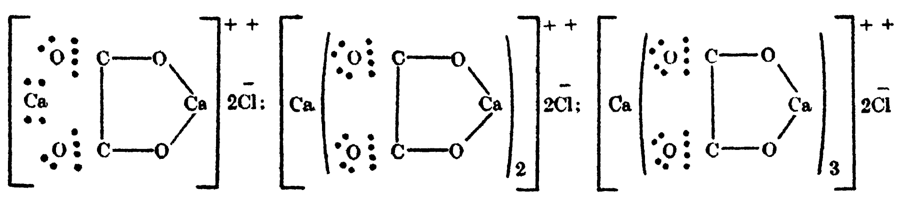
or



Here again the neutralisation of charges results in the chlorine ions being held to oxygen by oxonium valencies. The view might be advanced that the chlorine ions are attached to the calcium by a co-valency, thus giving the calcium a co-ordination number of four, but the existence of calcium chloride addition compounds of calcium oxalate of the formulae

$CaC_2O_4.CaCl_2.7H_2O$, $2CaC_2O_4.CaCl_2.24H_2O$ and $3CaC_2O_4.CaCl_2.8H_2O$

makes such a supposition unlikely. To be consistent these are pictured as follows, the electrons being represented by dots



where the calcium has, progressively, co-ordination numbers of 2, 4 and 6 [cf. Pfeiffer, 1922]. If the chlorine atoms in the last of these were united by a co-valency, calcium would have the unlikely co-ordination number of eight.

Sugden [1927] has recently discussed molecular compounds in terms of singlet linkages but his views do not appear to present any advantage over the preceding.

EXPERIMENTAL.

Preparation of glutamic acid. Gluten flour[1] (100 g.) was added to warm concentrated HCl (300 cc., d. 1·16) and the mixture boiled for 6 hours. After treatment with charcoal and filtration the solution was kept below 0° for 12 hours and deposited 28·9 g. of crude glutamic acid hydrochloride. This crop was recrystallised from 50 cc. of boiling 20 % HCl and gave 23·7 g. of hydrochloride, which was dissolved in 20 cc. boiling water and treated with charcoal; it was then filtered and 33 cc. HCl (d. 1·16) were added. 21·6 g. of pure glutamic acid hydrochloride were obtained. By concentration of the original mother-liquors under reduced pressure further crops of crude glutamic acid hydrochloride mixed with ammonium chloride were obtained, and these recrystallised from the mother-liquors of the first crop gave finally a further 8·9 g. of pure glutamic acid hydrochloride. The total yield was therefore 30·5 g. from 100 g. of gluten. (Found: Cl, 19·3; N, 7·8. Theory: Cl, 19·3; N, 7·6%.) The rotation of the pure hydrochloride was determined at different concentrations with the following results:

c	10·0	7·5	5·0	2·5	1·25	0·6	0·5	0·25
$[\alpha]_{5461}$	+28·63°	+28·25°	+27·45°	+26·85°	+26·28°	+23·54°	+22·87°	+20·59°

The hydrochloride (36·7 g.) was dissolved in 200 cc. of water and heated on the water-bath. Aniline (18·6 g. = 1 mol.) was added and to the clear solution 200 cc. of 95 % alcohol. On cooling 27·55 g. of glutamic acid separated. It was recrystallised from the minimum volume (250 cc.) of boiling water and gave 24·1 g. of pure glutamic acid crystallising in hexagonal plates. From the mother-liquors a further 1·8 g. was obtained. (Found: N, 9·4. Theory: N, 9·5 %.)

Salts of glutamic acid. The hydrobromide, described by Hlasiwetz and Habermann [1873] as crystalline conglomerates, separates in large hexagonal tablets on treatment of glutamic acid with slight excess of 48 % HBr. Like the hydrochloride it melts at 214°, but this depends on the rate of heating. (Found: Br, 35·1. Theory: Br, 35·0 %.) The rotation was determined at different concentrations in water.

c	10·0	7·5	5·0	3·0	2·0	1·0	0·5
$[\alpha]_{5461}$	+23·09°	+23·30°	+23·13°	+22·50°	+22·0°	+20·57°	+18·70°

The hydriodide crystallises similarly on dissolving glutamic acid in slight excess of constant boiling HI freshly distilled over red phosphorus. It melts with effervescence at 180–185°. (Found: I, 45·7; N, 5·1. Theory: I, 46·1; N, 5·1 %.) The only reference to this salt appears to be a crystallographic one by Kaplanova [1917].

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[1] Purchasable from Messrs Bishop and Brooke, 21 Cock Lane, Snowhill, E.C.

The rotations of the salt were determined at various concentrations.

c	10·0	7·5	5·0	3·0	2·0	1·0	0·5
$[\alpha]_{5461}$	+18·64°	+18·39°	+18·35°	+18·14°	+17·59°	+15·79°	+11·57°

If the effect of variation of the concentration of the three halogen acid salts be plotted against specific rotation the curves shown in Fig. 2 are obtained.

There is a rapid falling off of specific rotation in solutions below 3 % due to hydrolysis of the salts, the values for dissociated salt approaching that of glutamic acid. Exact confirmation of this is found in the work of Wood [1914] who observed a similar phenomenon for several amino-acids when specific rotation was plotted against the ratio of the number of molecules of hydrochloric acid to molecules of amino-acid. If the specific rotation of the glutammonium ion be calculated from each salt at a dilution where hydrolysis is negligible there is substantial agreement between the observations.

Salt	$[\alpha]_{5461}$ ion
Glutamic acid hydrochloride	+33·4°
Glutamic acid hydrobromide	+34·6°
Glutamic acid hydriodide	+33·4°

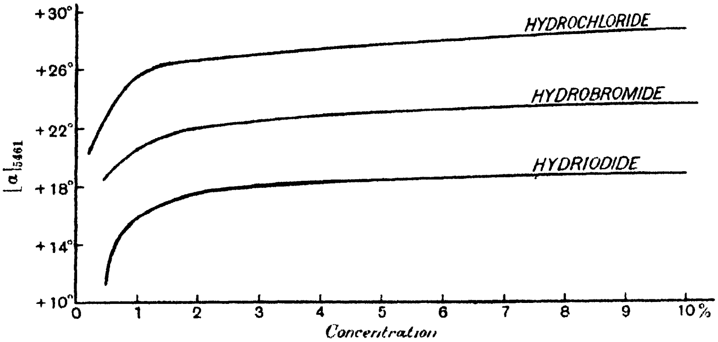

Fig. 2

Calcium glutamate. Prepared from pure glutamic acid and calcium carbonate, this salt crystallises slowly from syrupy solutions in small hexagonal plates which are readily soluble in water.

0·2000 g. dried at 160° lost 0·0073 g. $H_2O = 3·7$ %.
0·2052 g. dried at 160° lost 0·0087 g. $H_2O = 4·2$ %.
0·1927 g. dry; $NH_3 = 12·01$ cc. *N*/10 acid. N – 8·7 %.
0·1783 g. dry; 0·0751 g. $CaSO_4$. Ca = 12·4 %.
Calc. for $(C_5H_8O_4N)_2Ca.H_2O$; $H_2O = 5·1$ %: for $(C_5H_8O_4N)_2Ca$; Ca – 12·1 %; N = 8·4 %.

The rotations were determined over a wide range of concentrations.

c	30·0	25·0	20·0	15·0	10·0	5·0	2·5
$[\alpha]_{5461}$	+0·81°	–0·34°	–1·71°	–2·62°	–3·84°	–5·05°	–5·22°

Strontium glutamate. This salt crystallises very readily from concentrated solutions and separates in hexagonal plates.

0·2105 g. dried at 95° lost 0·0353 g. $H_2O = 16·8$ %.
0·2945 g.; 0·1152 g. $SrSO_4$. Sr = 18·7 %.
0·1752 g. dry; $NH_3 = 8·85$ cc. *N*/10 acid. N = 7·1 %.
Calc. for $(C_5H_8O_4N)_2\,Sr.4H_2O$; Sr = 19·3 %; $H_2O = 16·0$ %: for $(C_5H_8O_4N)_2\,Sr$; N = 7·4 %.

It gave the following specific rotations:

c	10·0	5·0	2·5
$[\alpha]_{5461}$	−2·96°	−3·59°	−3·95°

Barium glutamate. This salt crystallises in irregular hexagonal plates from concentrated solutions.

0·2093 g. dried at 160° lost 0·0376 g. $H_2O = 18·0$ %.
0·2040 g.; $NH_3 = 7·87$ cc. *N*/10 acid. N = 5·4 %.
0·1975 g.; 0·0881 g. $BaSO_4$. Ba = 26·2 %.
Calc. for $(C_5H_8O_4N)_2\,Ba.5\frac{1}{2}H_2O$; $H_2O = 18·0$ %; N = 5·3 %; Ba = 26·2 %.

The following specific rotations were obtained:

c	10·0	5·0	2·5
$[\alpha]_{5461}$	−2·44°	−2·94°	−3·22°

Lithium glutamate. This salt crystallises in irregular hexagonal plates which are anhydrous.

0·1959 g.; $NH_3 = 12·43$ cc. *N*/10 acid. N = 8·9 %.
Calc. for $C_5H_8O_4N\,Li$. N = 9·2 %.

The specific rotations of this salt were as follows:

c	10·0	5·0	2·5	1·25
$[\alpha]_{5461}$	−3·01°	−4·52°	−5·19°	−5·68°

Sodium glutamate. A standard solution was made up from a known weight of pure glutamic acid and standard alkali. The specific rotations were determined at a variety of concentrations.

c	15·0	10·0	5·0	2·5	1·25
$[\alpha]_{5461}$	−2·57°	−3·57°	−4·30°	−4·67°	−4·75°

Addition compounds of alkaline earth glutamates.

Calcium glutamate calcium chloride. Glutamic acid hydrochloride (9·2 g.) was boiled with 50 cc. water and excess of calcium carbonate; the solution was filtered and concentrated. On keeping, the dihydrate of calcium glutamate calcium chloride (5·8 g.) separated in hexagonal plates. It also crystallises in needles. To avoid the known decomposing action of water on certain addition compounds, the crystalline crops were in every case filtered on hardened paper without washing and then rubbed dry on porous plate before analysis.

0·1138 g. dried at 160° lost 0·0086 g. $H_2O = 7·6$ %.
0·2046 g.; 0·1211 g. AgCl. Cl = 14·7 %.
0·2062 g.; $NH_3 = 8·7$ cc. *N*/10 acid. N = 6·0 %.
Calc. for $(C_5H_8O_4N)_2\,Ca.CaCl_2.2H_2O$; Cl = 14·8; N = 5·8; $H_2O = 7·5$ %.

It was unchanged by recrystallisation from water in which it is readily soluble (1 in 2·5 parts).

0·2024 g.; 0·1191 g. AgCl. Cl = 14·6 %.

On recrystallisation from solutions containing 2 or 5 molecules excess of calcium chloride, the same compound was obtained in each case.

2 molecules excess—0·1886 g. (anhydrous); 0·1265 g. AgCl. Cl = 16·6 %.
5 molecules excess—0·1746 g. (anhydrous); 0·1206 g. AgCl. Cl = 17·1 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaCl_2$; Cl = 16·0 %.

The specific rotations of solutions of the addition compound at a variety of concentrations were determined with the following results:

c	33·8	26·9	21·0	14·9	10·0	5·0	3·0	2·0	1·3
$[\alpha]_{5461}$	+0·9°	−0·18°	−1·13°	−2·02°	−2·7°	−3·40°	−3·50°	3·38° −	−3·56°

Calcium glutamate calcium bromide. Glutamic acid hydrobromide (11·4 g.) was boiled with excess of calcium carbonate in 50 cc. water; the solution was filtered and concentrated. The addition compound separated on keeping in hexagonal plates (6·9 g.).

0·2060 g. dried at 160° lost 0·0145 g. H_2O = 7·0 %.
0·1917 g.; 0·1255 g. AgBr. Br = 27·9 %.
0·1927 g.; NH_3 = 7·06 cc. N/10 acid. N = 5·1 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaBr_2.2H_2O$; Br = 28·0 %; N = 4·9 %; H_2O = 6·3 %.

The addition compound gave the following specific rotations:

c	15·0	10·0	5·0	3·75	1·9
$[\alpha]_{5461}$	−2·07°	−2·45°	−2·95°	−3·0°	−3·0°

Calcium glutamate calcium iodide. Prepared similarly to the preceding, this addition compound crystallised in irregular hexagonal plates.

0·1997 g. dried at 160° lost 0·0140 g. H_2O = 7·0 %.
0·1997 g.; 0·1380 g. AgI. I = 37·8 %.
Calc. for $(C_5H_8O_4N)_2$ Ca.$CaI_2.2\frac{1}{2}H_2O$; I = 37·8 %; H_2O = 6·7 %.

Barium glutamate barium chloride. Glutamic acid hydrochloride (9·2 g.) was dissolved in water, made alkaline with baryta and the boiling solution was saturated with carbon dioxide On filtration and concentration the addition compound (11·3 g.) separated in irregular hexagonal plates.

0·2020 g. dried at 160° lost 0·0299 g. H_2O = 14·8 %.
0·1721 g. (anhydrous); 0·0794 g. AgCl. Cl = 11·4 %.
0·1712 g. (anhydrous); NH_3 = 5·91 cc. N/10 acid. N = 4·8 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$BaCl_2$; Cl = 11·1 %; N = 4·4 %: for $(C_5H_8O_4N)_2$ Ba.$BaCl_2.6H_2O$; H_2O = 14·5 %.

The following specific rotations were obtained at various concentrations:

c	18·0	15·0	10·0	5·0	4·0	3·0	2·0
$[\alpha]_{5461}$	−1·38°	−1·62°	−1·90°	−2·45°	−2·49°	−2·25°	−2·47°

Barium glutamate barium bromide. Prepared similarly to the above, glutamic acid hydrobromide (11·4 g.) gave 8·4 g. of the addition compound crystallising in minute hexagonal plates which were *anhydrous.*

0·1896 g.; 0·0963 g. AgBr. Br = 21·6 %.
0·2018 g.; NH_3 = 5·9 cc. N/10 acid. N = 4·1 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$BaBr_2$; Br = 22·0 %; N = 3·9 %.

On recrystallisation from water a *tetrahydrate* was obtained.

0·2040 g. dried at 160° lost 0·0191 g. H_2O = 9·4 %.
0·1894 g. (anhydrous); 0·0942 g. AgBr. Br = 21·7 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.$BaBr_2.4H_2O$; H_2O = 9·0 %.

The specific rotations of the anhydrous salt at various concentrations were as follows:

c	20·0	15·0	10·0	5·0	4·0	3·0
$[\alpha]_{5461}$	−1·41°	−1·74°	−2·03°	−2·38°	−2·34°	−2·49°

Barium glutamate barium iodide. Prepared similarly to the preceding, this addition compound separated in irregularly shaped crystals.

0·1938 g. dried at 120° lost 0·0252 g. $H_2O = 11·4$ %.
0·1686 g. (anhydrous); 0·0977 g. AgI. I = 31·3 %.
Calc. for $(C_5H_8O_4N)_2$ Ba.BaI_2; I = 30·9 %: for $(C_5H_8O_4N)_2$ Ba.BaI_2.6H_2O; $H_2O = 11·6$ %.

Strontium glutamate strontium chloride. Glutamic acid hydrochloride (9·2 g.) was boiled in 50 cc. water with excess of strontium carbonate; the solution was filtered and concentrated. On keeping, a large crop (6·9 g.) separated of homogeneous appearance and consisting of microscopic narrow leaflets. It proved on analysis to be mainly strontium glutamate. (Found: Cl = 1·2 %.) The mother-liquors on concentration deposited tablets (1·8 g.) which were evidently crude addition compound (Cl = 9·6; N = 3·6 %, whence Cl : N = 1·07 : 1). On further concentration the liquors gave crude strontium chloride (1·7 g.) crystallising in long needles. (Found: Cl = 36·6 %.)

The first crop on recrystallisation from 12 cc. of boiling water gave pure strontium glutamate crystallising in rectangular plates.

0·2042 g. dried at 95° lost 0·0350 g. $H_2O = 17·1$ %.
0·1692 g. (anhydrous); NH_3 equivalent to 8·84 cc. *N*/10 acid. N = 7·3 %.
Calc. for $(C_5H_8O_4N)_2$ Sr; N = 7·4 %: for $(C_5H_8O_4N)_2$ Sr. 4½H_2O; $H_2O = 17·1$ %.

The pure addition compound was obtained as follows. Glutamic acid hydrochloride (4·6 g.) was treated with 50 cc. *N* hydrochloric acid, made alkaline by addition of strontia and the boiling solution was saturated with carbon dioxide. On filtration and concentration the addition compound separated in bold tablets. Preliminary essays showed that even with this excess of strontium chloride, strontium glutamate tended to separate first.

0·2047 g. dried at 160° lost 0·0642 g. $H_2O = 31·4$ %.
0·2047 g.; 0·0802 g. AgCl. Cl = 9·7 %.
0·2102 g.; NH_3 = 5·84 cc. *N*/10 acid. N = 3·9 %.
0·1965 g.; 0·0980 g. $SrSO_4$. Sr = 23·8 %.
Calc. for $(C_5H_8O_4N)_2$ Sr.SrCl_2.12H_2O; Cl = 9·4 %; N = 3·7 %; Sr = 23·2 %; $H_2O = 28·7$ %.

Strontium glutamate strontium bromide. Glutamic acid hydrobromide (2·8 g.) was treated in aqueous solution with excess of strontia and the boiling solution saturated with carbon dioxide. On filtration and concentration microscopic needles (2·1 g.) of crude strontium glutamate (found: N, 5·5 %; Br, 3·3 %) separated. The mother-liquors, now relatively richer in strontium bromide, deposited on concentration a homogeneous crop of needles, quite different in appearance from the first crop, which proved to be the required addition compound.

0·2020 g.; 0·0919 g. AgBr. Br = 19·4 %.
0·2123 g.; NH_3 = 4·7 cc. *N*/10 acid. N = 3·1 %.
Calc. for $(C_5H_8O_1N)_2$ Sr.SrBr_2.11H_2O; Br = 19·4 %; N = 3·4 %.

Strontium glutamate strontium iodide. A solution made up in the usual way from 3·4 g. glutamic acid hydriodide deposited 2·35 g. crude strontium glutamate (found: I = 3·9 %; N = 5·5 %). The mother-liquors, now relatively richer in strontium iodide deposited on further concentration well-formed hexagonal plates (0·55 g.) of the required addition compound.

0·1560 g.; 0·0863 g. AgI. I = 29·9 %.
0·2215 g.; NH_3 = 5·00 cc. *N*/10 acid. N = 3·2 %.
Calc. for $(C_5H_8O_4N)_2$ Sr.SrI_2.$7H_2O$; I = 29·9 %; N = 3·3 %.

Lithium glutamate and lithium halides. From solutions of glutamic acid hydrochloride, hydrobromide or hydriodide and lithium carbonate only crude lithium glutamate was isolated (found in the three cases respectively: Cl = 1·2 %; Br = 1·9 %; I = 3·9 %).

Calcium chloride and glutamic acid hydrochloride. Solutions of glutamic acid hydrochloride containing 2 or 5 molecular proportions of calcium chloride deposited crude glutamic acid hydrochloride. (Found: Cl = 22·0 %; 23·9 % respectively.)

Calcium chloride and glutamic acid. When glutamic acid was crystallised from a solution containing two molecular proportions of calcium chloride it was recovered contaminated with a small proportion of chloride. (Found: Cl = 2·0 %.)

Addition compounds of alkaline earth aspartates.

Strontium aspartate strontium chloride. Aspartic acid (2·66 g.), prepared from asparagine, was dissolved in 20 cc. hot *N* hydrochloric acid (1 mol.) and boiled gently with excess of strontium carbonate. On filtration and slight concentration the solute crystallised readily as a micro-crystalline powder (1·85 g.).

0·2110 g. dried at 160° lost 0·0128 g. H_2O = 6·1 %.
0·1990 g.; 0·1346 g. $SrSO_4$. Sr = 32·3 %.
0·2011 g.; 0·0975 g. AgCl. Cl = 12·3 %.
0·1937 g.; NH_3 = 6·84 cc. *N*/10 acid. N = 4·9 %.
Calc. for $(C_4H_6O_4N)_2$ Sr.$SrCl_2$.$2H_2O$; Sr = 32·1 %; Cl = 13·0 %: N = 5·1 %: H_2O = 6·6 %.

On concentration the mother-liquors deposited the same addition compound (1·25 g.).

Barium aspartate and barium chloride. Aspartic acid (1·33 g.) was treated with 100 cc. 0·1 *N* hydrochloric acid (1 mol.), made alkaline with baryta and then carbon dioxide was passed into the boiling solution in excess. The filtered solution was concentrated to about 10 cc. and the compound crystallised in microscopic narrow leaflets of homogeneous appearance, yield 1·7 g. On analysis it gave Ba = 35·8 %, Cl = 5·0 %; N = 5·2 %, corresponding to a mixed crystal containing barium chloride and barium aspartate in the ratio 1 : 2·6 respectively.

When a similar quantity of aspartic acid was subjected to the same series of operations but using three molecular proportions of hydrochloric acid, the crystalline product consisted of microscopic rhomb-shaped plates, yield 1·7 g. On analysis it gave Ba = 39·6 %, Cl = 7·6 %, N = 5·2 %, corresponding to a mixed crystal, this time relatively richer in barium chloride, containing barium chloride and barium aspartate in the ratio 1: 1·7 respectively.

Calcium aspartate and calcium chloride. No crystalline product could be obtained from equimolecular proportions of these compounds in water.

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CLVII. THE PHOSPHORUS OF CASEINOGEN.

I. ISOLATION OF A PHOSPHORUS-CONTAINING PEPTONE FROM TRYPTIC DIGESTS OF CASEINOGEN.

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The work described in this paper was commenced in 1924, and a preliminary report issued in the following year [Rimington and Kay, 1925], in which it was stated that from tryptic digests of caseinogen a preparation had been obtained containing amino-acids in association with phosphoric acid, and representing approximately 10 % of the organic phosphorus of the original digest.

Since that time, experimental work has been directed along two main lines of approach. In the first place an investigation of the behaviour of caseinogen towards various proteolytic enzymes, phosphatases, acids and alkalis was made [Rimington and Kay, 1926; Rimington, 1927, 1], from which it was possible to draw certain conclusions relative to the state of combination of phosphorus in the caseinogen molecule. The linkage involved appears to be of the ester type, phosphoric acid being united to some hydroxyl-containing constituents of the protein molecule, most probably hydroxyamino-acids. A study of artificially phosphorised proteins [Rimington, 1927, 1] afforded additional evidence in support of this view.

At the same time experiments were being continued having as their object the isolation in a pure state of the phosphorus-containing substance present in tryptic digests of caseinogen. This has now been accomplished, and it is with this isolation that the present paper is concerned.

ISOLATION OF A PHOSPHORUS-CONTAINING PEPTONE.

The liberation from caseinogen, during the action of trypsin, of an acid-soluble, organic substance containing all the phosphorus of the protein has been noted by Biffi [1898], Plimmer and Bayliss [1906] and others, but neither Dr Kay nor myself was aware, when these experiments were begun, that any serious attempt had been made to isolate this substance. Our attention has since been called by Posternak [1927] to a Swiss patent taken out by the Society of Chemical Industry in Basle [1923] in which the separation of a preparation containing organically combined phosphorus from caseinogen digests is described. A discussion of this and of Posternak's later work will be found below.

In an earlier communication it was shown that the liberation of the soluble organic substance by trypsin occurs with great rapidity, the subsequent splitting off of phosphoric acid taking place at a much slower rate. Isolation at the period when the soluble organic phosphorus was at a maximum did not however yield the best results as the quantity of peptide material present in these early stages was relatively great. By trial it was found that the best time to commence operations was 38–40 hours after the addition of trypsin. In illustration of the process, the details of an actual preparation are given.

1 kg. of caseinogen was digested with 500 cc. strained extract of pancreas in the presence of toluene for 38 hours at 37° and a p_H of 8·4 to 8·6, the total volume of liquid being 11 l. Glacial acetic acid was then added to bring the p_H to 4·5 and the whole filtered through fine linen. The filtrate (11·1 l.) contained 4·2 g. organic phosphorus.

1·8 l. of saturated lead acetate and 700 cc. of ammonia were then added in the order mentioned, the supernatant liquid removed and the precipitate filtered off. After repeated washing with water, it was suspended in water and decomposed by hydrogen sulphide. The filtrate, freed from hydrogen sulphide by a stream of air, was rendered alkaline with ammonia and magnesia mixture added with vigorous stirring. Precipitated magnesium ammonium phosphate was filtered off.

The filtrate was now adjusted to p_H 3·8 by means of acetic acid and hot saturated uranium acetate solution added until no further precipitation occurred. The precipitate was filtered off, washed thoroughly, and suspended in water, to this suspension sufficient 10 % sulphuric acid being added to effect complete solution. Well cooled 40 % sodium hydroxide was then added with vigorous stirring until all the uranium was precipitated. This was then filtered off by suction into flasks containing a little acetic acid in order to neutralise the filtrate immediately, since the substances sought are unstable in alkaline solution. No other method of removing the uranium was found to be practicable, but it was possible to carry out the whole of the above process, from the addition of the sodium hydroxide to the collection of the filtrates, within 5 minutes.

The uranium-free filtrates were combined, the resulting liquid having a slightly acid reaction. It possessed an N/P ratio of approximately 5/1, gave a strong biuret reaction (red) but no Millon and no glyoxylic reactions. A solution of phosphotungstic acid produced no precipitate.

The insoluble copper salt was next prepared by the addition to the solution of sufficient saturated copper acetate to produce complete precipitation. About 2 l. of copper acetate solution were required, the total volume amounting to 5 l.

This precipitate was filtered off, washed and dried over sulphuric acid *in vacuo* (copper precipitate I): weight 55·5 g.; total phosphorus 4·81 %; total nitrogen 7·50 %; ratio N/P = 3·45: yield 65 % of the organic phosphorus of the digest. For this stage the above yield is typical, although on one occasion as much as 74 % of the original organic phosphorus of the digest was obtained.

This substance, whose ratio N/P did not differ greatly from one of the preparations described by Posternak [1927], was not yet pure as was shown by fractional precipitation with copper acetate in the following manner.

15 g. copper precipitate I, N/P = 3·45, were suspended in water and decomposed by hydrogen sulphide. After filtration, using well washed "fuller's earth[1]" to remove the last traces of colloidal copper sulphide, the liquid was again treated with saturated copper acetate solution, 280 cc. being required to produce complete precipitation. The resulting precipitate (copper precipitate II) was separated, washed and decomposed as before. The solution was now treated with copper acetate in successive small quantities, the precipitates produced at each addition being separately removed. A fractionation was thus achieved, the four precipitates, referred to as copper precipitate III *A*, *B*, *C* and *D* respectively, being analysed for phosphorus and nitrogen with the following results.

Table I.

Cu ppt. III	Weight g.	Phosphorus %	Nitrogen %	N/P
A	1·72	6·34	7·44	$\frac{2\cdot60}{1}$
B	5·50	5·50	7·32	$\frac{2\cdot95}{1}$
C	2·53	5·32	7·43	$\frac{3\cdot09}{1}$
D	2·22	5·09	7·30	$\frac{3\cdot18}{1}$
Total recovered	11·97			

It is evident that the greater part of the material, amounting to 67 % of the total quantity, comes down in the middle fractions (*B* and *C*) with a ratio N/P = 3. Repeated reprecipitation and fractionation of this material failed to alter its N/P ratio. It was therefore considered to be homogeneous and analysed[2] with the following result, from which the empirical formula $(C_{37}H_{53}O_{33}N_9P_3)Cu_{4\cdot5}$ was deduced. It contained no sulphur.

	C	H	O	N	P	Cu
Calculated	28·98	3·62	34·44	8·22	6·07	18·66
Found (mean of duplicate analyses)	28·63	4·18	34·68	7·84	6·05	18·62

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[1] "Fuller's earth" proved to be a very effective adsorbent for colloidal copper sulphide but to retain not more than traces of the phosphorus-containing substances.

[2] The analysis recorded in this and the following paper [1927] have been carried out either by Mr F. Flack of the Chemical Laboratory, or by Mr A. R. Colwell, of the Biochemical Laboratory, Cambridge or by "Fein Chemie," Tübingen.

The purified substance represented just under 50 % of the original organic phosphorus of the caseinogen digest. It was a light blue powder, insoluble in water but yielding solutions, when the copper was removed by hydrogen sulphide, which gave an intense red biuret reaction. The preparation of the free acid in the dry state is described below.

The two fractions *A* and *D*, obtained during the purification of the crude material, were investigated in order to ascertain whether they contained other peptones in addition to that described above. The material *A* had a ratio N/P = 2·6 not far removed from that required by a substance containing 8 nitrogen atoms to 3 of phosphorus. Upon analysis, however, it was found to be a mixture of the peptone already described and copper phosphate. The fraction *D* had a ratio N/P = 3·18, whilst that of the filtrate from the precipitation was 3·4, indicating that whatever impurity is present at this stage is there only in small quantity. All attempts to isolate from these fractions pure material having a higher ratio of nitrogen to phosphorus than that of the peptone described were without success. There appears to be some phosphorus-free material which follows the precipitation of the phosphorus-containing peptone rather closely, and it seems possible that this may account for the variety of preparations with varying N/P ratios found by Posternak [1927].

PREPARATION AND PROPERTIES OF THE FREE PHOSPHOPEPTONE.

15 g. pure copper salt were decomposed by hydrogen sulphide and the resulting solution concentrated over sulphuric acid *in vacuo* at room temperature. There resulted a highly viscous syrup which solidified to a "glass." All efforts to obtain crystalline material were unsuccessful. Upon dehydration with absolute alcohol and pulverisation the peptone was obtained as a white, slightly hygroscopic powder, dissolving readily in water to yield distinctly acid solutions. Difficulty was experienced in effecting the combustion of this material, but on analysis it gave the following figures, which agree fairly well with the formula $C_{37}H_{62}O_{33}N_9P_3$ calculated from the composition of the copper salt.

	C	H	N	P
Found	37·44	5·75	10·13	7·05
Calculated	35·38	5·14	10·03	7·41

The amino-nitrogen, determined by the method of Van Slyke, was 1/9 of the total nitrogen; after hydrolysis with hydrochloric acid, however, the amino-nitrogen became equal to the total nitrogen. One would infer therefore that the substance is a peptone consisting of 9 amino-acids in peptide linkage, all of which are acyclic mono-amino-acids. An aqueous solution gave the following colour and precipitation tests:

Ninhydrin	+	Picric acid	–
Biuret	+ (deep rose)	Phosphotungstic acid	–
		Trichloroacetic acid	–
Glyoxylic	–	Lead acetate	dense white precipitate
Millon	–	Uranium acetate	voluminous yellow precipitate
Xanthoproteic	–	Copper acetate	blue precipitate
Sulphur test	–	Mercuric acetate	dense white precipitate
Molisch	–	Silver nitrate + ammonia	gelatinous white precipitate

It was noted in addition that its aqueous solutions gave a deep, orange-red colour with diazobenzenesulphonic acid and sodium carbonate, which turned golden yellow on addition of hydrochloric acid but left no secondary colour whatever after reduction with zinc dust and hydrochloric acid and addition of ammonia. The diazo-reaction could not therefore be due to histidine, which gives a yellow secondary colour; it was afterwards traced to hydroxyglutamic acid which is present in the peptone and which, as observed by Dakin [1918], yields the primary colours described. An investigation of the products of hydrolysis is presented in the following paper.

The colour tests demonstrate the absence of tryptophan, tyrosine, aromatic substances, carbohydrates, cystine and histidine. The high oxygen content on the other hand suggests that hydroxyamino-acids may be present together with phosphoric acid.

As has been previously reported [Rimington and Kay, 1926], investigation showed that the substance was slowly hydrolysed by pancreatic juice, phosphoric acid being produced and the peptide linkages also attacked. Phosphatases on the other hand bring about the separation of the phosphorus as phosphoric acid within a few hours. Sodium hydroxide (0·25 N) also liberates the whole of the phosphorus in inorganic form in 24 hours at 37°. Phosphopeptone is freely diffusible through collodion sacs.

Molecular weight. The determination of the molecular weight of the peptone could not be accomplished by any of the standard physico-chemical methods owing to its high degree of ionisation in solution, results being obtained which were very much too low. No better success was obtained when using the barium salt. The insolubility of the substance in camphor precludes employment of the method of Rast [1922]. From the analytical data, however, the ratio of amino-nitrogen to total nitrogen and from the action of bone phosphatase, which only removes 2/3 of the total phosphorus as phosphoric acid, it seems that the formulation as given is most likely correct. In view of the ready diffusibility of the substance it is assumed that the molecular weight is that indicated by the empirical formula, namely, 1254.

Optical activity. The peptone is strongly laevo-rotatory. The light used was the mercury green.

α in 2 dm. tube − 2·659°.

Nitrogen determination on solution 165·6 mg. per 100 cc.

$$[\alpha]^{15^\circ}_{5461} = -80{\cdot}53^\circ,$$

$$M[\alpha] = -1011{\cdot}53.$$

Basicity. The basicity was determined by titration, using 0·1 N sodium hydroxide and thymolphthalein as indicator.

0·1 g. free acid required 6·9 cc. sodium hydroxide, whence basicity = 8·7.

The result is in good agreement with the analysis of the copper and barium salts which show the peptone to behave as a nine-basic acid.

Brucine salt. It was hoped that the peptone might yield a crystalline salt with one of the alkaloids. To a strong aqueous solution of the free peptone, nine equivalents of brucine were added together with a little methyl alcohol to aid solution. The resulting solution was neutral in reaction. A slight excess of brucine was then added so that the mixture was alkaline to phenol red. No crystalline material separated, although within the course of a few hours an oil had precipitated. This was separated off, and allowed to stand for several months. It could not, however, be induced to crystallise but gave a glassy material which was ground, very thoroughly extracted with absolute alcohol, and the resulting powder dried *in vacuo* for analysis.

	N	P
Found	8·00	2·10
Calc. for $C_{244}H_{286}O_{64}N_{27}P_3$	8·02	1·98

It appears that the substance was the brucine salt of the peptone containing nine equivalents of brucine, with a molecular weight of approximately 4700.

Barium salt. The barium salt was prepared by adding a saturated solution of baryta to a concentrated aqueous solution of the peptone until faintly alkaline and then pouring into alcohol. The insoluble barium salt was centrifuged off, washed repeatedly with absolute alcohol and dried *in vacuo.* It formed a fine white powder very soluble in water.

Analysis $(C_{37}H_{53}O_{33}N_9P_3)Ba_{4\cdot5}$.

	N	P	Ba
Found	6·25	4·60	32·26
Calculated	6·76	4·99	33·14

DISCUSSION.

Since the proof by Hammarsten [1877] that phosphorus is an integral constituent of the caseinogen molecule, research has repeatedly been directed to the discovery of the type of linkage by which this element is held in combination. By analogy with sulphur, it might be supposed that there exists a phosphorus-containing amino-acid; however, the ready production of orthophosphoric acid from caseinogen under the action of various mild reagents has led to the view that it is phosphoric acid which is built in some way into the nitrogenous complex.

Among the early attempts to separate phosphorus-containing material from caseinogen those of Salkowski [1901], Levene and Alsberg [1901] and Reh [1907] may be mentioned. All of these workers however used as their starting material peptic digests in which an enormous quantity of peptide material was present. In no case was any substance isolated in a pure form. Posternak [1927] has lately reported the isolation of a variety of phosphorus-containing substances from tryptic digests of caseinogen. His experimental methods are not cited, nor are analytical data presented, so that it is difficult to form any opinion of his work. In the experience of the present writer material has several times been isolated which bore a close resemblance, from the point of view of elementary composition, to some of the preparations described by Posternak; in all cases however further work showed that these substances were still impure and that by suitable manipulation their components could be separated. In view of the above findings I find it difficult to believe that Posternak's preparations were homogeneous, and moreover I have been unable to confirm his statements regarding the structural units with which phosphoric acid is associated (see following paper). The alternative remains however, that the methods employed by him may have been so different from mine as to lead to dissimilar products. Of this, for the reasons stated above, I am unable to judge.

The phosphorus-containing complex present in tryptic digests of caseinogen appears to be largely, if not entirely, a phosphorus-containing peptone in which the phosphorus is present as phosphoric acid united by ester linkages to some or other of the amino-acids. The yield in which this purified peptone may be isolated corresponds to about 50 % of the original organic phosphorus of the digest. Although not entirely trypsin-stable, its peptide linkages are comparatively resistant to this enzyme: the phosphorus is also liberated with great slowness by trypsin. Phosphatases, on the other hand [Rimington and Kay, 1926], bring about a very rapid liberation of the phosphorus as phosphoric acid.

SUMMARY.

1. A phosphorus-containing peptone, $C_{37}H_{62}O_{33}N_9P_3$, has been isolated from tryptic digests of caseinogen in a yield corresponding to 50 % of the organic phosphorus of the digest.

2. It is a strongly acidic substance forming well-defined copper and barium salts each containing nine equivalents of the metal. It titrates as a nine-basic acid. A brucine salt has also been obtained. The free acid has $[\alpha]^{15°}_{5461} = -80{\cdot}53°$.

3. The substance, which has been designated "*phosphopeptone*," contains 1/9 of its nitrogen in the form of amino-nitrogen. After hydrolysis the ratio amino-nitrogen/total nitrogen becomes unity.

4. Colour tests indicate the absence of the usual amino-acids, the biuret and ninhydrin reactions are however positive. A primary colour reaction is also given with diazobenzenesulphonic acid, but there is no secondary colour after reduction.

5. The peptide linkages of phosphopeptone are slowly attacked by trypsin, which also brings about the slow liberation of the phosphorus as phosphoric acid.

In conclusion I wish to express my thanks to Sir F. G. Hopkins for the interest he has taken in this work. My best thanks are also due to Dr H. D. Kay for much valuable criticism and many helpful suggestions.

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CLVIII. THE PHOSPHORUS OF CASEINOGEN.

II. CONSTITUTION OF PHOSPHOPEPTONE.

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(*Received August 29th, 1927.*)

In the preceding paper [Rimington, 1927, 2] the isolation from caseinogen of a pure phosphorus-containing peptone has been described. This substance, which has been named *phosphopeptone*, and whose composition is represented by the formula $C_{37}H_{62}O_{33}N_9P_3$, has been isolated in a yield corresponding to 50 % of the organic phosphorus of the original tryptic digest. In the present paper evidence is adduced to show that the remaining organic phosphorus is present in similar if not identical form, and an account is given of the isolation and identification of the individual units of phosphopeptone, together with a suggested structure.

ISOLATION OF THE AMINO-ACIDS IN PHOSPHOPEPTONE.

20 g. of phosphopeptone were hydrolysed by boiling with 20 % hydrochloric acid for 36 hours. The resulting solution was concentrated and saturated with hydrogen chloride. After 4 days in the ice-box and a second saturation no material had crystallised except a small quantity of ammonium chloride. Hydrochloric acid was removed from the liquid by repeated concentration *in vacuo*; the resulting syrup was then diluted to about 200 cc. and the amino-acids were converted into their calcium salts by the method of Foreman [1914]. The phosphoric acid was at the same time converted into calcium phosphate and removed with the excess of lime by filtration.

After concentration *in vacuo*, the salts of the dibasic acids were precipitated by 97 % alcohol, about 2 l. being required. The sticky precipitate was dissolved in water and calcium removed quantitatively with oxalic acid. The solution was then concentrated and saturated with hydrochloric acid gas.

No glutamic acid hydrochloride could be separated even after long standing. Hydrochloric acid was therefore removed by repeated evaporation *in vacuo*, and the amino-acids converted into their copper salts by boiling with fresh copper hydroxide. The dark blue solution was concentrated and allowed to stand at 0° but nothing crystallised out. Aspartic acid was therefore absent.

Copper was removed from the solution by hydrogen sulphide and traces of chloride by acid silver nitrate, sodium hydroxide was then added to neutrality and the silver salt of hydroxyglutamic acid precipitated as described by Dakin [1918]. Thus obtained it was nearly pure, and, after one reprecipitation, the silver salt was analysed.

Analysis. $C_5H_7O_5NAg_2$.

	C	H	N	Ag
Found	15·42	1·75	3·5	57·5 %
Calculated	15·9	1·85	3·7	57·3 %

Yield: just over two molecules per molecule of phosphopeptone. For further identification the copper salt was prepared by boiling a solution of the acid, prepared from the silver salt, with excess of freshly precipitated copper hydroxide, filtering, concentrating, and precipitating the copper salt by alcohol.

Analysis. $C_5H_7O_5NCu$.

	C	H	N	Cu
Found	25·1	3·50	5·8	27·4 %
Calculated	26·7	3·12	6·2	28·3 %

As the copper salt is not very suitable for analysis and as difficulty was experienced in getting the silver salt quite free from silver oxide, the free acid was also prepared by decomposing 1·5 g. of the silver salt with hydrogen sulphide and concentrating the resulting solution over sulphuric acid *in vacuo* at room temperature. It was obtained (0·7 g.) in microcrystalline form and corresponded with Dakin's [1918] description, giving all the colour tests described by him. When heated it softened indefinitely at about 100°. For analysis a sample was dried over phosphorus pentoxide at 35–40°.

Analysis. Ash = 1·2 %.

	C	H	N
Found (ash-free)	36·5	5·60	8·60 %
Calc. for $C_5H_9O_5N$	36·8	5·52	8·59 %

The only dibasic acid present in the peptone is hydroxyglutamic acid. Its nitrogen corresponds to between two and three atoms out of the total of nine in the molecule.

The alcoholic solution of the calcium salts of the monobasic acids was concentrated *in vacuo* and calcium was removed by oxalic acid. The resulting solution gave a precipitate with neutral lead acetate, which proved to be only lead oxalate, due to a slight excess of oxalic acid in the solution. No lead salts were precipitated from the filtered solution by the addition of ammonia. Lead was removed from the filtrate as sulphide. The resulting liquid was concentrated to a small bulk *in vacuo* at 37° and the concentration continued over sulphuric acid *in vacuo* at room temperature. A syrup was obtained which gradually deposited a fairly large quantity of crystalline material.

Under the microscope two types of crystal were visible; in the greater proportion, very small platelets or prismatic needles, frequently grouped together in dense aggregates, and among these large, rather irregular plates similar in appearance to crystals of serine.

A method of separation based upon chemical properties was sought without success. An attempt was therefore made to achieve separation by fractional crystallisation of the remainder of the material.

Differences in solubility were not great, but the more abundant substance was eventually obtained in a fairly pure state. It was very hygroscopic and had to be dried to constant weight over phosphorus pentoxide *in vacuo* at 50° before analysis. The figures obtained agree most nearly with the formula $C_4H_9O_3N$ which is that of hydroxyaminobutyric acid, a homologue of serine.

Analysis (micro.).

	C	H	N
Found	41·18	8·83	11·67 %
Calc. for $C_4H_9O_3N$	40·34	7·56	11·77 %

The copper salt was prepared by boiling a solution of the acid with freshly precipitated copper hydroxide. On concentration the liquid deposited small prismatic crystals having an intense deep blue colour. The copper salt of hydroxyaminobutyric acid was found by Schryver and Buston [1926] to contain two molecules of water of crystallisation.

Analysis. $(C_4H_8O_3N)_2Cu.2H_2O$.

	N	Cu
Found	8·60	19·73 %
Calculated	8·34	18·93 %

The dibenzoyl derivative was prepared by adding 3 cc. benzoyl chloride and 7 cc. 5*N* sodium hydroxide to a solution of 0·5 g. of the acid in 10 cc. water. After acidification, the precipitated product was extracted repeatedly with boiling light petroleum to remove benzoic acid and the insoluble residue was crystallised from a little hot benzene. It crystallised in fine needles having M.P. 109°. Schryver gives the M.P. as 112°.

The second fraction was small in amount and was consequently more difficult to obtain in a pure state. It crystallised in flat plates, was hygroscopic and was analysed after desiccation over phosphorus pentoxide.

Analysis. Nitrogen 12·60 %; Ash 2·83 %; Nitrogen (ash-free) 12 97 %.

The nitrogen content of serine is 13·33 %.

The substance melted with decomposition at 223°. The M.P. of *l*-serine is 224°. It also gave a benzoyl derivative which crystallised in fine needles, M.P. 121°. Dibenzoylserine melts at 124°.

Lack of material prevented further methods of identification, but from the above data it appears probable that the substance under investigation was an impure specimen of serine.

No other amino-acids were found.

The difficulty of separating the hydroxyamino-acids from one another and from other amino-acids is well known. In the above case the yield of hydroxyglutamic acid corresponded to between two and three molecules per molecule of phosphopeptone, but the yields of hydroxyaminobutyric acid and of serine were considerably smaller.

The possibility exists that phosphopeptone may contain some hitherto unknown amino-acid, nevertheless, assuming that only the three which have been isolated are present, namely, hydroxyglutamic acid, hydroxyaminobutyric acid and serine, in the relative proportions depicted below, a formula can be arrived at for the peptone which agrees with that indicated by elementary analysis.

The molecule of phosphopeptone would thus appear to be made up of the following:

Hydroxyglutamic acid	3 molecules
Hydroxyaminobutyric acid	4 ,,
Serine	2 ,,
Phosphoric acid	3 ,,

Hydroxyaminobutyric acid has so far been isolated from only one protein, namely, from oat glutelin by Schryver and Buston [1926]. The above scheme would require its presence in caseinogen to the extent of 1·8 % or, if the whole of the phosphorus is similar to phosphopeptone, of about 3·6 %. Similarly the serine would amount to 0·8 % or 1·6 % of the protein. The best yield of serine from caseinogen so far recorded is 0·5 % but the amount actually present is undoubtedly greater [cf. Emil Fischer, 1903].

CONSTITUTION OF PHOSPHOPEPTONE.

In endeavouring to form a picture of the way in which the units of this peptone are united certain requirements, determined by experiment, must be fulfilled, namely:

(i) The molecular formula shall be $C_{37}H_{62}O_{33}N_9P_3$.

(ii) The substance must behave as a nine-basic acid.

(iii) One-ninth of the total nitrogen shall be present in the form of amino-nitrogen, the remaining linkages must be peptide linkages.

(iv) An explanation must be offered of the fact that two-thirds only of the total phosphorus is removed by bone phosphatase in the form of phosphoric acid, the remaining one-third being however almost certainly also in ester linkage as it is removed by the phosphoric esterase of kidney extracts [see Rimington and Kay, 1926].

Whilst it must be emphasised that as yet there is no possible means of deciding to *which* of the constituent amino-acids the phosphorus atoms are attached, nor in what sequence they occur, a structural formula can be constructed which satisfies *all* of the above conditions. This formula is depicted in Fig. 1 but it must be remembered that the positions of the amino-acids and phosphorus atoms are chosen arbitrarily.

It will be seen that two of the molecules of phosphoric acid are visualised as being united through one hydroxyl group only to the amino-acid chain, whilst the third forms a di-ester linkage. This affords a ready explanation of the difference between the action of bone and of kidney phosphatase, a fact which was difficult to interpret upon any other basis[1].

Subsequent work may demonstrate the exact positions of the phosphorus groups, but in the meantime it was thought advisable to give the above formula for the sake of descriptive clearness.

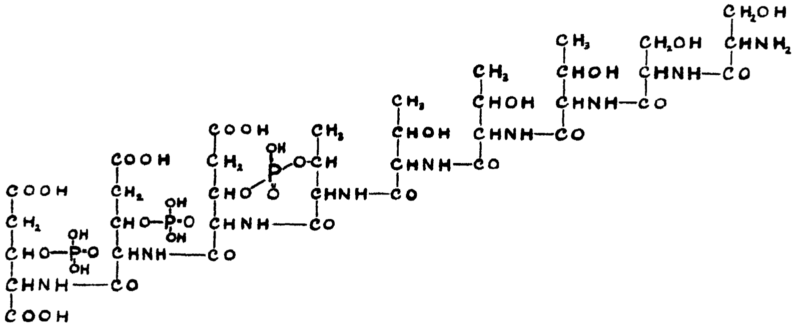

Fig. 1.

(3 molecules of hydroxyglutamic acid) (4 molecules of hydroxyaminobutyric acid) (2 molecules of serine

THE RELATION OF PHOSPHOPEPTONE TO THE REMAINDER OF THE CASEINOGEN PHOSPHORUS.

As has been pointed out, the yield of pure phosphopeptone prepared from tryptic digests of caseinogen represents 50 % only of the original organic phosphorus. When however the action was investigated of bone and kidney phosphatase upon phosphopeptone and upon neutralised tryptic digests respectively a very striking similarity was observed. In both cases only two-thirds of the total organic phosphorus was hydrolysed by bone phosphatase preparations, the remainder being liberated by the kidney enzyme. These results are shown in the two figures previously published [Rimington and Kay, 1926, p. 783, Fig. 4; Rimington, 1927, 1, p. 278, Fig. 2].

It appears probable therefore that if other substances analogous to phosphopeptone exist, the form of the phosphorus linkages in them must be similar to those in phosphopeptone itself. The peptide linkages of phosphopeptone appear to be fairly resistant to tryptic hydrolysis; on the other hand, the peptone is split off from the rest of the caseinogen molecule with such rapidity that the linkages between it and the parent grouping must be of a labile nature and readily attacked by trypsin.

To the above considerations is due, no doubt, the fact that the material isolated from different preparations has, when pure, always the same composition.

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[1] Martland and Robison [1927] have recently shown that bone phosphatase has very little action upon the diethyl ester of phosphoric acid while the monoethyl ester is readily hydrolysed.

DISCUSSION.

The association of phosphoric acid in the caseinogen molecule with a complex of hydroxyamino-acids raises several interesting questions. No evidence is as yet available concerning either the specific metabolic importance of the hydroxyamino-acids as a class, or the nutritional significance, if any, of phosphoric acid united with them in such a compound as the phosphopeptone just described. The behaviour of this peptone as a whole, its ready diffusibility and resistance to trypsin in no way exclude the possibility of its absorption from the gut into the blood-stream unchanged.

Its ready hydrolysis by the enzymes of ossifying cartilage also makes the suggestion not improbable that the phosphorus of caseinogen may by this means contribute in part to the inorganic phosphate laid down in bone formation. Experiments bearing upon this aspect of the problem are now in progress.

Caseinogen has frequently been regarded as a conjugated protein, the phosphorus it contained having been assumed to be present in some prosthetic group. The association seen in phosphopeptone cannot be regarded as of this kind, *i.e.* one in which constituents of a non-protein nature are characteristically present.

During the course of proteolysis, fragments, some large and some small, are formed by cleavage of the protein under the action of the enzyme. Some of these fragments show comparative resistance towards further hydrolysis, and phosphopeptone, however built into the general architecture of the caseinogen molecule, must be regarded as of this type.

Many proteins other than caseinogen contain phosphorus, although usually to a much smaller extent. It is, however, impossible to state whether or not in these cases also the association is one of phosphoric acid with hydroxyamino-acids. If such should prove to be the case, it might be permissible to regard the combination as a new unit constituent of proteins in general, such as, for example, phosphorylglutamic acid, which would be related to hydroxyglutamic acid in much the same way that cysteine is to serine.

SUMMARY.

1. From phosphopeptone after hydrolysis have been isolated hydroxyglutamic acid, hydroxyaminobutyric acid and serine.

2. From the above data a hypothetical structural formula has been constructed that is in agreement with all other known chemical properties of the peptone.

In conclusion I wish to thank Sir F. G. Hopkins for the interest he has taken in this work and also Dr H. D. Kay for encouragement and advice.

Note in conjunction with H. D. Kay.

S. Posternak recently called our attention [Posternak, 1927, 1] to work of his own on a patented process for "preparing the phosphorus nucleus of caseinogen" which had appeared (unaccompanied by the author's name) in a Swiss Patent Specification in 1923. Although we had examined the literature, this specification had unfortunately escaped our notice. Lately [1927, 2] he has claimed to have isolated from caseinogen a pure phosphopolypeptide which contains the following amino-acids: glutamic acid, 3 mols.; aspartic acid, 0·6 mols.; *iso*leucine, 3 mols.; serine, 3 mols.; phosphoric acid, 4 mols. From these findings he comes to the rather surprising conclusion that the phosphorus nucleus of caseinogen is composed of four serine-phosphoric acids.

Knowing some of the difficulties and pitfalls attending this work of purification and analysis, we are of opinion that it is only in the light of considerably more experimental and analytical detail than is presented by Posternak that his conclusions regarding the chemical composition of the "phosphorus nucleus of caseinogen" can be assessed.

If we take his statements at their face value we have no alternative but to conclude that the phosphopeptone first obtained by us in a crude form in 1924 (described at a meeting of the Biochemical Society on Feb. 9th, 1925 [Rimington and Kay, 1925]), and which has now been purified and analysed by one of us, is entirely different from the "pure" substance described by Posternak.

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CLIX. OBSERVATIONS ON SOME PROCESSES OF OXIDATION IN BLOOD-SERUM.

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(*Received August 5th, 1927.*)

The usual method for the estimation of the amount of oxygen combined as oxyhaemoglobin in a sample of blood consists in treating the fluid with potassium ferricyanide in faintly alkaline solution, when a complicated reaction occurs in which methaemoglobin is formed and an amount of oxygen equal to that originally present as oxyhaemoglobin is set free for measurement. But that the change which takes place when ferricyanide is added to so complicated a mixture as blood is completely expressed by this simple statement has long been questioned. Among the more recent work bearing on this point we may mention Conant and Fieser's [1924] demonstration of the dependance of the completeness of the reaction between potassium ferricyanide and oxyhaemoglobin on the degree of alkalinity of the medium in which that reaction takes place, and also the results of Smith, Dawson and Cohen [1920] which prove that in the van Slyke method of applying the ferricyanide reaction—a method in which the liberated oxygen is extracted at once from the reaction mixture by exposure to a vacuum—an appreciably greater amount of oxygen is liberated from a given quantity of blood than is obtained by the procedure of Henderson and Smith, in which the blood and ferricyanide are merely shaken together in a known volume of air and the amount of oxygen liberated is measured by the resulting increase in the percentage of oxygen in the gas phase. But apart from these and earlier investigations of the completeness of the reaction between ferricyanide and oxyhaemoglobin the observation has also been made that, when the oxygen has been set free in this way, if the shaking and exposure be continued, the liberated gas is slowly re-absorbed by the reaction mixture. This phenomenon is most marked with blood from anaemic patients, and with such blood it may proceed at such a rate as to render impossible any accurate estimation of the volume of oxygen originally liberated. The effect is so definite and of such magnitude that it seemed desirable to make an attempt to obtain some more precise knowledge as to the factors and substances involved when a mixture of blood and potassium ferricyanide absorbs oxygen under these conditions, and the results recorded in the present paper represent the information that we have collected.

For these experiments we used a compact form of Haldane's [1920, 1922] new blood-gas apparatus that is represented diagrammatically in Fig. 1. The main features are that the long rubber tubes of the original form of the apparatus are eliminated, and that the whole apparatus can be immersed in a thermostat bath through the glass sides of which the readings are taken. The zero line of the U-tube manometer is drawn on a small mirror which is arranged so that it is adjustable in a vertical direction in order to allow for slight variations in the level at which the manometric liquid (xylene) stands. The gas-measuring burette has a total volume of 0·2 cc. and is graduated in 0·002 cc. so that using mercury as confining fluid readings of gas volume correct to ± 0·0002 cc. can be obtained. This degree of accuracy is, however, far greater than that required for the purposes of this particular piece of work.

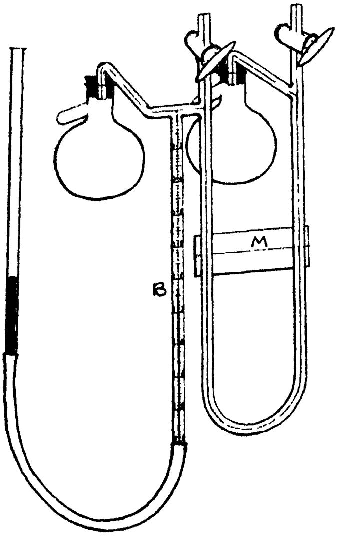

Fig. 1. Compact form of Haldane blood-gas apparatus. *B* is the gas-measuring burette containing mercury as confining fluid, and *M* the adjustable mirror carrying the zero line for the manometer U-tube.

The apparatus is most conveniently mounted on a plate of aluminium about $\frac{1}{8}$ in. thick with a suitable slot for receiving the mirror and its clamping screw.

Our ordinary procedure has been to place 1 cc. of the blood or other material under investigation together with 2 cc. of dilute ammonia solution (4 cc. of a solution of d. = 0·880 diluted to 1 l.) or other alkaline solution in the reaction-bottle and to place a similar mixture containing 1 cc. of distilled water in place of the blood or serum on the control side. We used in the side-tube of each bottle 0·5 cc. of a saturated solution of potassium ferricyanide, and from the final colour of our reaction mixtures it was evident that this always constituted a great excess. The bottles having been fitted to the rest of the apparatus, the whole was lowered into the thermostat and when temperature equilibrium had been attained the taps were closed and the ferricyanide tilted into the experimental material. The apparatus was shaken by hand for 2 minutes and then replaced in the bath for a further 3 minutes. By adjustment of the mercury reservoir tube the level of the xylene was brought to the zero line on the mirror and the reading of the gas volume then noted. This gave a starting-point for the observations of the subsequent absorption of oxygen, which was followed usually for about an hour by shaking the apparatus for 2 minutes and allowing it to rest in the bath for the following 3 minutes before each reading. By this procedure we, of course, systematically neglected the amount of oxygen absorption occurring during the first 5 minutes of each experiment; we did this in order to avoid occasional irregularities due to the mixing of the materials or to evolution of gas from small quantities of haemoglobin present, and all the curves we give are plotted from results consistently obtained in this way. But we have convinced ourselves that no appreciable difference is produced by the inclusion of these initial 5-minute intervals in the diagrams and that our conclusions are therefore in no way affected either qualitatively or quantitatively thereby. All our experiments were carried out at room temperature, which rarely varied outside the limits of 15–18°, and all the gas volumes recorded in our diagrams have been reduced to N.T.P.

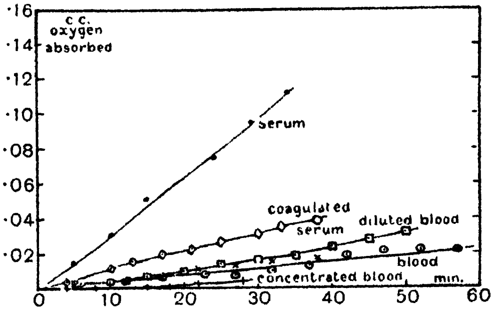

Fig. 2. Effect of variation of the concentration of corpuscles on the rate of absorption of oxygen by blood in the presence of ferricyanide. The points denoted by crosses were obtained in the presence of NaF. The oxidation curve for coagulated serum is also given.

With this technique it was easy to show, in the first place, that the absorption of oxygen by a mixture of defibrinated blood and ferricyanide is due entirely to the serum and that the corpuscles play no part in it. For example in Fig. 2 we record the results of observations made on the blood of a male patient, S., who possessed a haemoglobin content of 60 % of normal and a red cell count of $3{\cdot}44 \times 10^6$ per mm.^{3}. It will be seen that doubling the concentration of the corpuscles by centrifuging almost completely abolishes the absorption of oxygen, while dilution of the blood with an equal volume of its own serum very considerably increases it. The rate at which the pure serum itself takes up oxygen in the presence of ferricyanide is very considerable, amounting to about 0·1 cc. per cc. of fluid in 30 mins. It is evident, then, that we are not here dealing with an absorption of oxygen by immature red corpuscles such as might have been expected to occur in the blood of an anaemic patient, and that the oxidation is so much more marked in this than in normal blood simply because the anaemic blood contains the larger proportion of serum. As a matter of fact the rate of this oxygen absorption in the whole untreated blood even of this patient is such as might easily be overlooked in an ordinary oxygen determination, and in the blood of a normal person would be even less. With the serum from this same blood we convinced ourselves that absorption of oxygen does not occur in the absence of ferricyanide, obtaining a volume difference of only 0·0005 cc. during 25 minutes' shaking of the serum alone in air. We also showed that this oxidation is not dependent on a process of glycolysis inasmuch as the rate of absorption of oxygen by 1 cc. of this blood was totally unaffected by the addition of 0·2 cc. of 0·5 % sodium fluoride solution according to Evans' recommendation [1922] (see the points represented as crosses in Fig. 2). We further obtained evidence that we were not dealing with an enzyme reaction of any kind by observing the rate of absorption of oxygen by 1 cc. of serum that had been heated by holding the bottle in boiling water for 5 mins. before the experiment. Certainly in this case the rate of oxidation was much reduced (Fig. 2), but this is readily to be explained by the slow rate of diffusion and the difficulty of adequate shaking caused by the presence of large masses of coagulum.

In considering the various constituents of serum that might be responsible for the formation of autoxidisable substances under the influence of potassium ferricyanide we first of all suspected that the effect might be due to sugar. But we found that, under the conditions of our experiment—that is, in faintly alkaline solution—1 cc. of 0·5 % glucose solution absorbed no appreciable amount of oxygen when shaken with air in the presence of ferricyanide, the actual change in volume observed at the end of an experiment lasting 41 mins. amounting to only 0·0008 cc.

We were left, therefore, with the alternative possibilities that the autoxidisable constituents arise either from the proteins of the serum, or from the lipins, or possibly from a colloidal complex of the two. We therefore attempted to fractionate the serum in various ways and to study the oxidisability of the separate fractions. We tried, for example, to extract the lipins by shaking with ether, and found that the residual aqueous fluid showed a much slower rate of oxygen absorption than the original serum, and that this reduction of oxidisability was the more marked the more efficient the ether extraction. When the ether extract was evaporated to dryness in a dish floating on warm water a residue was obtained that turned brownish during the last stages of the evaporation. When this residue was re-emulsified in some of the extracted serum no appreciable increase in the rate of oxidation was produced, undoubtedly because of oxidation of the lipins during the evaporation. When we repeated the experiment in such a way that the ether extraction and also the evaporation of the extract were carried out in an atmosphere of nitrogen, we found that when the lipin residue obtained was re-emulsified in the extracted serum the rate of oxygen absorption was markedly increased. The result of this experiment is recorded in Fig. 3 in which it is to be noted that the curve labelled "extracted serum + lipins" was obtained with 1 cc. of extracted serum to which the lipins obtained from 1·5 cc. of the original serum had been added, so that in this experiment the effect of the addition of the lipins is exaggerated. In passing we would call attention to the particularly rapid rate of absorption of oxygen shown in this experiment by the mixture of untreated serum and ferricyanide, 0·24 cc. oxygen being taken up by 1 cc. serum in less than 50 mins. This is associated with the circumstance that the blood used in this experiment was obtained from a patient, F., exhibiting so marked a degree of lipaemia that the serum was opaque and milky with the excessive amount of fat it contained. We have confirmed these effects of the ether extraction and re-addition of lipins using serum containing only a normal content of these substances; we have found further that extraction with light petroleum also reduces to a similar extent the rate at which the serum will absorb oxygen in the presence of ferricyanide. The fact that extraction with these lipin solvents reduces the oxidisability of the serum does not necessarily indicate that the lipins themselves are concerned in the effect, for, these solvents produce a certain amount of precipitation of the serum-proteins. This effect is not very marked in the case of ether, but with light petroleum emulsion formation is very troublesome, and vigorous shaking of the mixture of serum and petroleum results in an emulsion of the consistence of vaseline, which separates only imperfectly into petroleum and aqueous layers in the centrifuge, a compact mass of material giving protein reactions separating between the two main liquids. The reduction of the oxidisability of the serum described in these experiments might therefore well be due to an effect of the extracting fluid on the proteins, but the fact that the re-addition of the extracted lipins does in some measure increase the rate of oxidation of the extracted serum can be interpreted only as indicating a direct participation

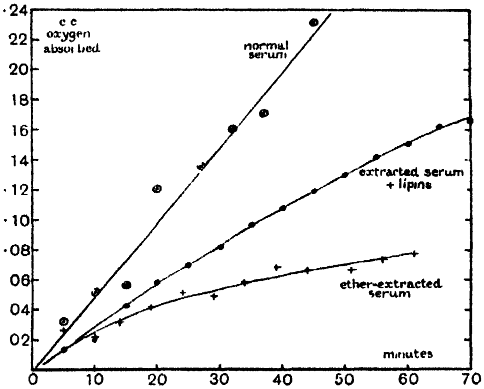

Fig. 3. Effect of ether extraction and re-addition of the lipins obtained by evaporation of the extract out of contact with air.

of the lipins in the process of oxidation here under study. By re-addition of the extracted lipins, however, we have never succeeded in restoring the rate of oxidation to that shown by the original unextracted serum, but whether this incompleteness of the restoration of the oxidisability is due merely to incompleteness of mechanical dispersion we are at present not able to say.

In view of the evidence that exists in favour of the view that the lipins of the serum are present in the form of colloidal complexes with the proteins, we carried out also an experiment on the separation of the proteins and lipins of the serum by the method described by Hardy and Gardiner [1910]. Serum was run into some six times its volume of alcohol previously cooled to − 8°; the precipitated proteins were filtered off at a temperature not rising above 0°, then freed from alcohol by thorough washing with ether and finally dried in the vacuum desiccator. One half of this material was dissolved in a volume of water equal to half that of the serum originally taken, and the oxidation of this solution in the presence of ferricyanide was observed. The lipins were recovered from the combined filtrates by evaporation *in vacuo*. The residue was emulsified by shaking in a volume of distilled water equal to that of the original serum, the process being conducted in an atmosphere of hydrogen. One half of the resulting emulsion was used for an oxidation experiment, while in the other the remaining half of the protein precipitate was dissolved by shaking in hydrogen to yield a "resynthesised" serum whose oxidisability was also studied. The results of this experiment are shown in Fig. 4 from which it will be seen that both the protein and the lipin fractions take up oxygen slowly in the presence of ferricyanide, and that the mixture of the two, in the proportions and concentrations in which they originally existed in the serum, absorbs oxygen at a rate hardly greater than that to be expected from the arithmetical summation of the effects due to the two constituents separately. From this it would seem that both proteins and lipins are concerned in the process of oxidation and that neither exerts an appreciable accelerating influence on the oxidation of the other. If a lipin-protein complex is re-formed under the conditions of our experiments it does not show any appreciably greater susceptibility to oxidation than is possessed by its separate lipin and protein constituents. It will be noticed, however

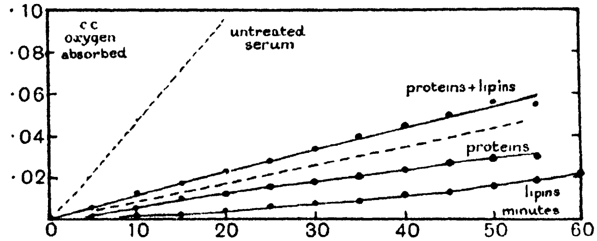

Fig. 4. Rate of oxidation in the presence of ferricyanide of the proteins and lipins separated from serum by ice-cold alcohol. The unlabelled dotted line represents the arithmetical sum of the curves for lipins and proteins respectively.

how far we were in this experiment from achieving a re-synthesis of the original serum by a re-union of its separated constituents: it is evident that the process of separation had produced deep-seated and irreversible changes of the colloidal structure of the serum and consequent reduction in the rate of oxidation—an effect apparently analogous to the diminution of the rate of oxygen absorption observed by Warburg [1914] to follow successive destruction of structure in nucleated red blood cells.

Our next observations are concerned with the fractionation of the serum by means of ammonium sulphate. A first experiment showed that when serum is saturated with this salt the oxidisable substances are completely precipitated—the filtrate absorbing no oxygen at all when treated with ferricyanide in the presence of ammonia according to our usual procedure.

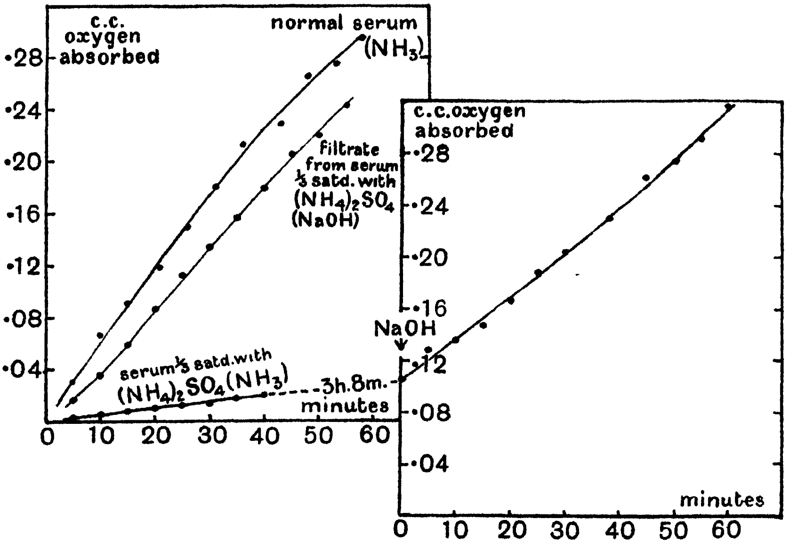

Fig. 5. Effect of one-third saturation with ammonium sulphate and subsequent addition of NaOH. The alkali present during the oxygen absorption is mentioned in brackets at the end of the description of each curve.

But the precipitated proteins were also practically devoid of oxidisable properties when treated in this way. This result suggested that possibly the large amount of ammonium sulphate present in these experiments had depressed the ionisation of the ammonia used as alkali to such an extent that the final mixture was far less alkaline than in our previous experiments, and that therefore the oxidising potential of the ferricyanide was so much reduced that it was incapable of giving rise to the autoxidisable substances whose behaviour we are here studying. It is possible also that the rate of absorption of oxygen by these autoxidisable substances themselves might have been reduced by a reduction in the alkalinity of the medium. We therefore proceeded to the experiment whose results are recorded in Fig. 5. In this case we compared the rate of absorption of oxygen by untreated serum in the presence of ammonia with that shown by serum mixed with one-third its volume of saturated ammonium sulphate solution. In this mixture the rate of oxygen absorption was very slow until 1 cc. of approx. *N* NaOH solution was added at the point indicated. The result of the increased alkalinity was shown in an immediate increase in the rate of oxidation to a value not far different from that of the untreated serum. Evidently, then, the depressant effect of the ammonium sulphate is to be ascribed to the reduction it produces of the alkalinity of the reacting mixture. In Fig. 5 we include also the results of an experiment on the *filtrate* from serum one-third saturated with ammonium sulphate, and examined in the presence of 1 cc. *N* NaOH instead of ammonia. It is evident that one-third saturation with ammonium sulphate does not precipitate any appreciable amount of the substance or substances responsible for the oxidations we are studying—a result confirmed by experiments described below.

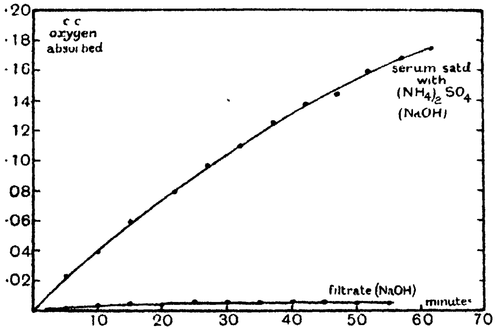

Fig. 6. Precipitation of the oxidisable materials by full saturation of the serum with ammonium sulphate.

On repeating now the experiment with full saturation with ammonium sulphate and using 1 cc. *N* NaOH as alkali it was easy to show that this procedure precipitated completely the oxidisable substances, for the filtrate from serum saturated with ammonium sulphate showed no oxygen uptake even in strongly alkaline solution, while the unfiltered mixture of serum and ammonium sulphate under the same conditions absorbed oxygen at a rate commensurate with that observed with the untreated serum (Fig. 6). The oxidisable material was therefore contained in the precipitate. A further series of observations illustrating the successive precipitation of the active material by ammonium sulphate is recorded in Fig. 7, from which it will be seen that, as mentioned above, one-third saturation with the salt removes only a small portion of the oxidisable substances while half saturation brings down a much larger fraction. In all these experiments we used such an amount of each of the various filtrates as corresponded to 1 cc. of the original serum, and added ammonium sulphate solution to the reaction mixtures in such amounts that the concentration of this substance, together with the amount of NaOH and the total volume of the liquid under test, was the same for all experiments of any one series, so that they are strictly comparable *inter se*. As we were able to confirm the results of Fig. 7 on another sample of serum we may state definitely that the oxidisable substances are precipitated completely by full saturation with ammonium sulphate, partially by half saturation and hardly at all by one-third saturation with ammonium sulphate.

Seeing that from the results of the ether extraction experiments we concluded that the lipins of the serum are to a large extent concerned in the oxidations we are considering, we were naturally led to compare the behaviour of lecithin emulsions towards ammonium sulphate with that of the serum itself. It is easy to show that a 1 % emulsion in distilled water of Merck's lecithin (prepared from eggs) is rapidly and completely precipitated by saturation with ammonium sulphate, less rapidly but still as completely by half saturation and that even one-third saturation with the salt will throw out the whole of the lipin in the space of 2 to 3 hours. As the liquids from which the results of Fig. 7 were obtained were allowed to stand overnight before filtering, it might have been expected that the precipitation of the lipins would have been complete even in the case of the serum only one-third saturated with ammonium sulphate. But it is also easy to show that the presence of proteins very much modifies the conditions of precipitation of lecithin by salts. For example, if a mixture of 1 cc. of a 1 % emulsion of lecithin lightly stained with Sudan III and 1 cc. of 1 % gelatin solution be added to 10 cc. $N/100$ NaOH and the solution be then one-third saturated with ammonium sulphate by the addition of 6 cc. of the saturated solution, it is evident from the red colour remaining in the liquid that the bulky precipitate that is produced does not contain the whole of the lipin, although, in the absence of the protein, lecithin itself is completely precipitated under these conditions. In this alkaline solution the protein exerts a protective action on the lecithin. If we can assume that similar relationships obtain between the proteins and the lipins of the serum we can at once account for our observations.

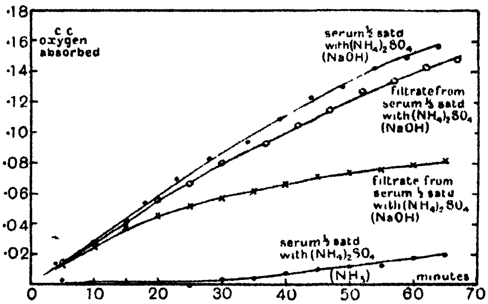

Fig. 7. Progressive precipitation of the oxidisable materials by increasing concentrations of ammonium sulphate.

These results have a bearing on the general question of the relationship of lipins to proteins in serum. As Chick demonstrated [1914], the euglobulin of serum is a complex of pseudoglobulin and phospholipin and is precipitated by one-third saturation of the serum with ammonium sulphate. That this procedure does not render the serum lipin-free is evidently due to the fact that the serum-proteins exert a protective action on the lipins similar to that which we have observed to be exerted on lecithin by gelatin: we can thus account for Chick's observation that after precipitation of the euglobulin from serum by one-third saturation with ammonium sulphate there remains sufficient lipin in the filtrate to combine slowly with pseudoglobulin to form a further quantity of euglobulin.

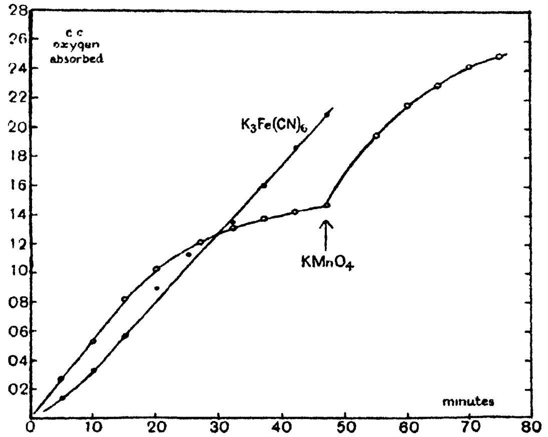

Fig. 8. Effect of substitution of potassium permanganate for ferricyanide: at the arrow a second portion of permanganate was added to the serum.

We should mention lastly that we made a few experiments to determine the effect of substituting other oxidising agents for the ferricyanide used in all the experiments so far described. As is seen in Fig. 8, for example, potassium permanganate in dilute ammonia solution acts even more powerfully in promoting the oxidation than ferricyanide, but the effect gradually passes off owing to the fairly rapid reduction of the permanganate by the proteins and other oxidisable constituents of the serum. On adding a further quantity of permanganate the rate of oxygen uptake is once more increased. So far as we are aware there are no experiments on the oxidation of lecithin in the presence of potassium permanganate with which we can compare this result obtained with serum. With potassium dichromate, which from the observations of Thunberg [1916] is known to accelerate the oxidation of ordinary lecithin, we obtained no acceleration of the oxidation of serum comparable with that produced by ferricyanide and permanganate, and this no matter whether we worked in an alkaline medium (1 cc. *N* NaOH + 1 cc. saturated ammonium sulphate being added to 1 cc. of the serum) or in an acid medium.

in which case 2 cc. approx. 0·1 *N* acetic acid were added to 1 cc. of serum (Fig. 9). Using ferric chloride also we found no appreciable absorption of oxygen to take place in the acid medium, but when the alkaline mixture was used the rate of oxygen uptake after a short initial delay due probably to the difficulty of efficient shaking of the mixture before the bulky precipitate of ferric hydroxide had shrunken together, was no less rapid than in the presence of ferricyanide (Fig. 9). This result again is just the opposite of that obtained by Warburg and Meyerhof [1913] with emulsions of ordinary lecithin, for they found that iron was a more efficient catalyst of the oxidation in acid solution than in neutral or alkaline.

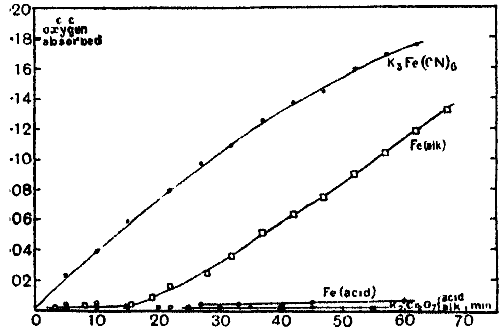

Fig. 9. Substitution of ferric salts and potassium dichromate for ferricyanide in the oxidation of serum.

It is natural to compare the results of this investigation with those obtained by Harris [1926] in his work on the oxidation of blood-plasma exposed to light. He found that when exposed to light 1 cc. plasma absorbs oxygen at the rate of about 0·024 cc. per hour, and ascribed this effect to the tyrosine and tryptophan present in the molecules of the plasma-proteins. But as this rate of oxidation is of the order of one-tenth of that which we have observed in the serum in the presence of ferricyanide, and, further, as Harris found that the addition of ferricyanide produces a marked degree of protection of the plasma against oxidation under the conditions of his experiments, it is evident that the processes of oxidation that occur when plasma is exposed to light can account for only a small fraction of the total oxidation that we have observed in the serum in the presence of ferricyanide.

In conclusion we would add that we do not claim any measure of completeness for the data and explanations here presented, but we have thought it worth while to publish in this form the results we have obtained so far on account not only of their practical bearing on the technique of blood-gas analysis, but also of their relation to the general study of biological oxidation systems.

SUMMARY.

In the presence of certain oxidising agents such as potassium ferricyanide and potassium permanganate human blood-serum absorbs oxygen at the rate of about 0·25 cc. per cc. per hour when shaken with air at room temperature. The same effect is observed in the presence of ferric salts in alkaline solution, but not with ferric salts in acid solution nor with dichromate at any reaction. Extraction with ether reduces the rate at which the serum will absorb oxygen in this way, but this can be restored in some measure by the re-addition of the extracted material provided that this is recovered by evaporation of the ether extract in an oxygen-free atmosphere. This suggests that the substances primarily responsible for the effect are lipins and this explanation fits in with the further observations that we have made on serum fractionated by means of ammonium sulphate, when the conditions determining the precipitation of lipins by this salt in the presence of proteins are taken into account.

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CLX. THE SPECIFIC ACTION OF PLANT ENZYMES.

III. THE SPECIFIC CONDITIONS OF ACTION OF LEAF SALICINASES.

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WE have investigated the action of air-dried powdered leaves of some species of *Salix* and *Populus* on salicin solutions at various p_H values. The leaves of all the species of *Salix* and *Populus* (except *S. hastata*) were collected at Tashkend, dried rapidly at 25–30° and ground in a mortar to the state of fine powder. 0·2 g. of this powder was mixed with 10 cc. of McIlvain's [1921] buffer solution (citric acid and Na_2HPO_4) and with 5 cc. of 3 % salicin (Kahlbaum) solution. To all experimental flasks toluene was added and they were kept at 30–33°. For control the same conditions, but without salicin, were maintained. The determinations of glucose liberated were made by the Benedict-Lewis picric acid method, as we described in our previous paper [1925]. The p_H values were determined colorimetrically with Clark and Lubs' indicators. The full details are given for Exp. 1, but in all the other experiments only the difference between the amounts of reducing sugar formed in the presence and absence of salicin are quoted under the heading "Change in reducing sugar."

Exp. 1. The leaves of *P. Bachofenii* Wierzb., collected 13. vi. 1926. Duration of hydrolysis 44 hours.

p_H		Reducing sugar			
				Change	
Buffer added	At end	Experiment mg.	Control mg	mg.	%
3·0	3·0	3·5	2·0	1·5	12
3·4	3·6	5·0	2·2	2·8	22
3·8	4·2	6·0	2·0	4·0	31
4·2	4·6	8·5	2·1	6·4	50
4·6	4·9	12·0	3·8	8·2	64
5·0	5·2	14·0	4·3	9·7	76
5·4	5·3	11·5	3·5	8·0	63
5·8	5·8	17·0	4·3	12·7	100
6·2	6·4	15·0	4·3	10·7	84
6·6	6·5	15·0	4·3	10·7	84
7·0	6·9	15·0	4·6	10·4	82
7·4	7·1	13·5	4·6	8·9	70
7·8	7·3	10·5	3·8	6·7	53

Exp. 2. Leaves of *P. nigra* L. var. *pannonica*. Tashkend, 13. vi. 1926. Duration of hydrolysis 47 hours.

p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%
3·0	3·5	0·6	10
3·4	3·7	1·3	2·1
3·8	4·1	0·6	10
4·2	4·4	1·6	2·7
4·6	4·6	4·5	75
5·0	5·4	6·0	100
5·4	5·8	4·2	70
5·8	6·0	3·5	58
6·2	6·4	3·0	50
6·6	6·7	2·8	47
7·0	6·9	2·5	41
7·4	7·3	1·4	23
7·8	7·4	0·7	11

Exp. 3. Leaves of *P. candicans*. Tashkend, 13. vi. 1926. Duration of hydrolysis 19 hours.

p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%
3·2	3·6	0·0	0
3·8	4·6	0·1	6
4·2	4·9	0·15	10
4·6	5·4	0·35	23
5·0	5·5	0·5	33
5·4	5·7	0·1	7
5·8	6·0	0·75	50
6·2	6·4	1·5	100
6·6	6·6	0·8	53
7·0	6·9	0·25	17
7·4	7·2	0	0
7·8	7·4	0	0

Exp. 4. Leaves of *S. babylonica* L. Tashkend, 13. vi. 1926. Duration of hydrolysis 45 hours.

p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%
3·0	3·0	1·0	6
3·4	3·6	1·6	9
3·8	4·0	1·8	11
4·2	4·3	3·8	22
4·6	4·6	9·0	53
5·0	5·0	12·5	73
5·4	5·4	10·75	63
5·8	5·8	13·0	76
6·2	6·2	15·25	90
6·6	6·6	17·0	100
7·0	7·0	13·0	76
7·4	7·2	12·5	73
7·8	7·4	9·0	53

Exp. 5. Leaves of *S. oxica*. Tashkend, 13. vi. 1926. Duration of hydrolysis 46 hours.

p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%
3·0	3·2	0·1	2
3·4	3·7	0·5	8
3·8	4·2	0·6	10
4·2	4·6	1·0	17
4·6	4·9	2·7	46
5·0	5·0	3·5	60
5·4	5·4	3·0	51
5·8	5·8	3·6	61
6·2	6·2	4·6	78
6·6	6·5	5·2	89
7·0	6·9	5·9	100
7·4	7·2	3·0	51
7·8	7·3	2·3	39

Exp. 6. Leaves of *Salix* sp.? Tashkend, 13. vi. 1926. Duration of hydrolysis 44 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·2	0·4	4	5·8	5·7	8·5	89
3·4	3·6	0·5	5	6·2	6·0	9·5	100
3·8	4·1	0·9	9	6·6	6·5	8·5	89
4·2	4·5	1·5	16	7·0	6·9	8·4	88
4·6	4·9	2·6	27	7·4	7·2	3·8	40
5·0	5·2	3·1	32	7·8	7·4	1·5	16
5·4	5·4	4·3	46				

Exp. 7. Leaves of *S. hastata*. Biological Station (Murmansk), June 1926. Duration of hydrolysis 44 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	2·6	0	0	5·8	5·5	2·5	71
3·4	2·8	0	0	6·2	6·0	2·5	71
3·8	3·2	0	0	6·6	6·5	2·5	71
4·2	3·7	0	0	7·0	6·9	3·5	100
4·6	4·6	0	0	7·4	7·3	2·5	71
5·0	4·8	0·5	14	7·8	7·6	2·0	57
5·4	5·2	1·5	43				

Exp. 7. Leaves of *Gossypium hirsutum* L. The Turkestan Cotton Breeding Station No. 182, June 1926. Duration of hydrolysis 44 hours.

p_H		Change in reducing sugar (Experiment–Control)		p_H		Change in reducing sugar (Experiment–Control)	
Buffer	At end	mg.	%	Buffer	At end	mg.	%
3·0	3·4	0·4	10	5·8	5·8	4·1	100
3·4	3·8	0·8	20	6·2	6·2	3·1	75
3·8	4·2	0·6	15	6·6	6·6	2·4	58
4·2	4·6	0·8	20	7·0	6·9	2·2	54
4·6	4·8	2·3	56	7·4	7·2	1·9	46
5·0	5·0	3·5	85	7·8	7·4	1·4	34
5·4	5·4	2·9	71				

The optimal p_H values for the action of the salicinase of *Populus* are in general less than those for *Salix*, the greatest action of *Populus* salicinases being found at p_H 5·8–6·4 and that of *Salix* enzymes at p_H 6·0–6·9. It is of interest that the enzyme of *S. hastata* has a minimum of action at p_H 4·6–4·8 and the enzymes of middle-Asiatic species of *Salix* have a distinct action even at p_H 3·0–3·6. The values of salicin hydrolysis for this boreal species of *Salix* are generally less than for the others.

At p_H 5·3 for *P. Bachofenii*, 5·7 for *P. nigra*, 5·4 for *S. babylonica*, 5·4 for *S. oxica*, and 5·4 for *Gossypium hirsutum* it is possible to see a decrease in the hydrolysis of salicin by the enzyme. This decrease coincides probably with an isoelectric point of protoplasmic proteins, because the precipitation of proteins at their isoelectric points may carry down the enzymes and thus inactivate them. As the investigations of Pearsall and Priestley [1923] demonstrated, the most important proteins in tissues capable of growth have isoelectric points lying between p_H 3·0 and 6·0. The isoelectric points of potato-tuber tissues and of soya-bean root-tips were found by Robbins and Scott [1925] equal to p_H 6·4 and 6·2–6·44 respectively, for the mycelium of *Giberella saubinettii* the position of the isoelectric point is at p_H 6·2, for *Fusarium lycopersici* at p_H 5·5 and for *Fusarium oxysporum* at p_H 4·9. Pearsall and Ewing [1924] give for the isoelectric point of yeasts p_H 3·1–3·3 and for *Nitella* p_H 4·6–4·7.

The relative amounts of enzyme in the leaf tissues of the plants investigated were determined by special experiments with hydrolysis of salicin at p_H values optimal for each plant species. The duration of experiment was 43 hours at 31°.

	p_H (optimal)	Reducing sugar in mg.
P. Bachofenii	5·8	11·5
P. nigra v. *pannonica*	5·4	4·0
P. candicans	6·4	6·5
S. babylonica	6·6	16·5
S. oxica	6·9	5·5
S. sp.?	6·0	8·5
S. hastata	6·9	4·0
G. hirsutum	5·8	3·5

Similar relations are also revealed by experiments on the determination of the velocity constants of salicin hydrolysis by the various leaf enzymes. These experiments give the following results (the conditions as in previous experiments).

t — time in hours.

x — reducing sugar experimentally obtained.

y = reducing sugar calculated from the formula $k = \frac{1}{t} \cdot \ln \frac{a}{a-y}$.

P. Bachofenii

t	0	12	24	36	48	End
x	0	1·75	3·75	6·5	8·5	15·7
y	0	2·3	4·2	5·9	7·3	15·7

$k = 0·00571$

P. nigra

t	0	12	24	36	48	End
x	0	1·0	1·6	2·0	2·4	15·7
y	0	0·8	1·5	2·2	2·9	15·7

$k = 0·00187$

P. candicans

t	0	12	24	36	48	End
x	0	1·5	3·0	4·0	4·8	15·7
y	0	1·5	2·8	4·0	5·1	15·7

$k = 0·00358$

S. babylonica

t	0	12	24	36	48	End
x	0	4·5	9·5	12·5	15·5	15·7
y	0	5·6	9·2	11·6	13·0	15·7

$k = 0·01607$

S. oxica

t	0	12	24	36	48	End
x	0	1·3	2·5	3·2	4·3	15·7
y	0	1·2	2·4	3·4	4·4	15·7

$k = 0·00298$

S. sp.?

t	0	12	24	36	48	End
x	0	2·0	4·0	6·0	7·0	15·7
y	0	2·2	4·0	5·6	7·0	15·7

$k = 0·00535$

S. hastata

t	0	12	24	36	48	End
x	0	0·5	1·5	2·0	2·2	15·7
y	0	0·6	1·2	1·8	2·4	15·7

$k = 0·00150$

The coincidence of values obtained experimentally with those calculated from a monomolecular formula is very satisfactory.

SUMMARY.

1. A definite optimal hydrogen ion concentration exists for the action of salicinases from various species of *Populus* and *Salix*.

2. The different plants contain various amounts of the enzyme.

3. The presence of salicinase in *Gossypium hirsutum* confirms the view that this enzyme is not limited to the species of the Salicaceae, which was first expressed by Krauch [1880] who found salicinase in some Cucurbitaceae.

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CLXI. THE ACTION OF CARBON MONOXIDE ON CERTAIN OXIDISING ENZYMES.

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WARBURG [1926] has shown that the respiration of yeast is inhibited by carbon monoxide. Since this inhibition is prevented by light, and the combination of carbon monoxide with many complex iron compounds is known to be suppressed by light, he believes that the inhibition is due to the combination of the CO with the iron-containing "oxygen-transporting component of the respiratory ferment," which results in its inactivation. The action of carbon monoxide is thus analogous to that of cyanides.

Haldane [1927] has shown that carbon monoxide has a similar inhibitory effect on the activity of moths and cress plants, and has made it clear that this is very probably due to the poisoning of an oxidation-catalyst present in the cells.

Keilin [1927] has studied the action of CO on certain oxidising enzymes. He finds that the indophenol oxidases of both yeast and mammalian muscle are strongly inhibited by carbon monoxide and by cyanide, as is also the oxidation of catechol by the oxygenase of the potato.

In general then it would seem that carbon monoxide and cyanide act in a similar way; although the action of cyanide is usually the more marked, and fairly large amounts of carbon monoxide are necessary to produce a large inhibition (*e.g.* Warburg obtained a 60 % inhibition with 600 mm. pressure of CO).

The enzymes of the aldehyde-oxidase type (the dehydrases) have not so far been tested with carbon monoxide; and since the aerobic oxidation by certain of these systems is inhibited by cyanide, while that of others is completely unaffected, it will be interesting to determine whether carbon monoxide will show a similar distinction in behaviour towards these systems.

As convenient examples of these two classes of enzymes the aldehyde (and xanthine) oxidase of milk and the succinoxidase of muscle were chosen for investigation, since these enzymes can readily be obtained in solution free from other interfering enzymes by the methods of Dixon and Kodama [1926] and Ohlsson [1921] respectively. The oxygen uptake of the milk oxidase system has been shown by Dixon and Thurlow [1925] and by Dixon [1927] to be unaffected by cyanide; while Fleisch [1924] found that $M/1000$ cyanide was sufficient to prevent almost completely the uptake of oxygen by the succinoxidase system, leaving its power to reduce methylene blue unimpaired.

The experiments were carried out by means of the Barcroft differential apparatus at room temperature. The carbon monoxide was prepared from formate and sulphuric acid in the usual way, and was purified by passing it through strong NaOH, then through sulphuric acid, and finally through two long U-tubes filled with adsorbent charcoal. The Barcroft apparatus was filled with the appropriate gas mixture in the following manner. After the necessary solutions had been introduced into the apparatus it was placed in position in the thermostat with the taps open, and the open ends of the tubes were connected by means of rubber pressure-tubing and a glass T-piece with a tube communicating with a graduated mercury pressure gauge. This tube was also in communication, through taps, with a vacuum pump and with reservoirs of carbon monoxide and nitrogen. The apparatus could thus be exhausted until the pressure of air remaining, as shown on the gauge, corresponded with the desired pressure of oxygen. The tap connected with the vacuum pump was then turned off, and CO or nitrogen admitted into the apparatus. The final pressure was slightly above that of the atmosphere, as the gases were stored under slight pressure, and after shutting off the supply of gas the excess pressure in the apparatus was relieved by momentarily opening a fourth tap communicating with the atmosphere. (The outlet of this tap was kept just dipping into water to act as a trap.) The taps of the Barcroft apparatus were then immediately closed, the rubber tubes detached, and after shaking for five minutes in the water-bath readings were commenced. During the whole of the filling operation, which was usually complete in under one minute, the apparatus was gently shaken in the water-bath. The connecting tubes were of fairly narrow bore, so that their volume was small in comparison with that of the Barcroft apparatus; hence dead space effects were negligible, and the actual final pressures of the various gases inside the apparatus were equal (within 1 mm.) to those given by the gauge readings. Strong NaOH solution was placed in the small receptacles provided in the apparatus to absorb any traces of CO_2 which might be given off by the solutions. The same solutions were always added to both flasks of each apparatus, except that the substance to be oxidised (hypoxanthine or succinate) was added to the right-hand flask only. In the case of aldehyde, however, it was necessary to add this to both flasks, on account of its vapour pressure, so that in this case the enzyme solution was added to the right-hand flask only. The volume of liquid in each flask was in all cases made up to 3 cc. with buffer solution.

In Figs. 1 and 2 are given graphically the results of experiments on the aerobic oxidation of aldehyde and hypoxanthine by the milk oxidase. The curves show that carbon monoxide has no inhibitory effect in either case. Several experiments were carried out both with milk and with the oxidase preparation of Dixon and Kodama [1926] and the curves given are quite typical of the results obtained.

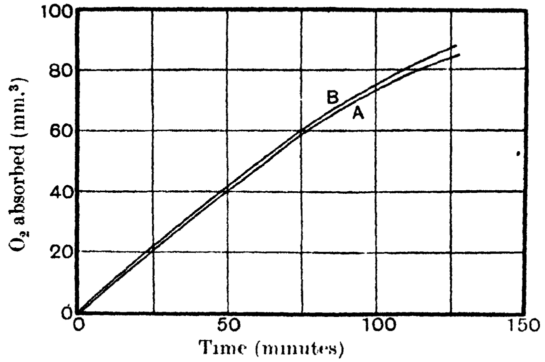

Fig. 1. Effect of CO on the Schardinger enzyme. Each apparatus contained 1 cc. fresh milk + 1 cc. phosphate buffer (p_H 7·5) + 1 cc. 0·5 % acetaldehyde. The composition of the gas in apparatus *A* was: O_2 5·3 %; N_2 94·7 %; and in apparatus *B*: O_2 5·3 %; N_2 21 %; CO 73·7 %. Temperature 16°.

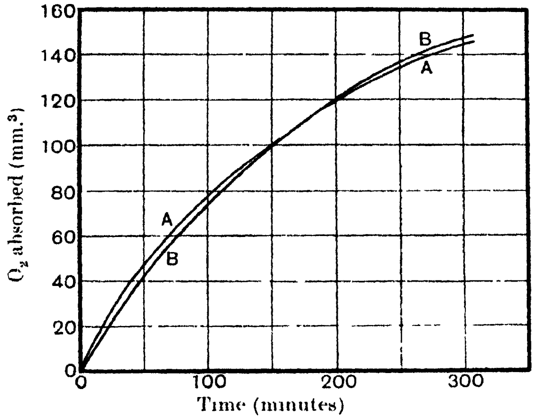

Fig. 2. Effect of CO on xanthine oxidase. Each apparatus contained 1 cc. oxidase solution + 1 cc. phosphate buffer (p_H 7·6) + 1 cc. (= 1 mg.) hypoxanthine solution. The compositions of the gas mixtures were: in *A*, O_2 5·2 %, N_2 94·8 %; in *B*, O_2 5·2 %, N_2 20·9 %, CO 73·9 %. Temperature 17°.

This negative result was to be expected, in view of the fact that cyanide has no action on these systems.

On the other hand, the fact that a small concentration of cyanide inhibits the aerobic (but not the anaerobic) oxidation of succinate by the succinoxidase would naturally be explained, on Warburg's view, by assuming the inactivation of the hypothetical oxygen-transporting iron complex present in the system; we might therefore expect that this system would show inhibition by carbon monoxide. The curves given in Fig. 3 show quite definitely however that this is not the case, the CO producing no inhibition whatever.

The enzyme solution used in this experiment was prepared from very fresh ox muscle by mincing the muscle, washing very thoroughly with distilled water, extracting with *M*/15 alkaline phosphate solution, and centrifuging the extract, in accordance with Ohlsson's [1921] method. The experiment was repeated a number of times with different enzyme preparations, and similar results were obtained. The very slight difference between curves *A* and *B* is clearly due to a small initial temperature difference; after the first few minutes the two velocities are identical throughout. Curve *C* shows for comparison the very striking inhibition produced by the addition of *M*/1000 cyanide.

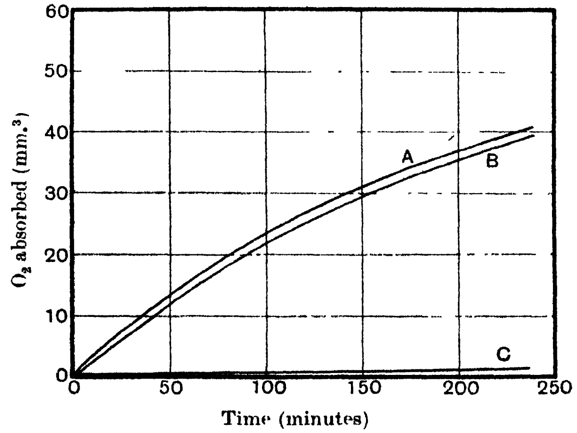

Fig. 3. Effect of CO on succinoxidase. Each apparatus contained 2 cc. succinoxidase solution (in phosphate p_H 8) + 1 cc. sodium succinate solution (= 2 mg. succinic acid). The compositions of the gas mixtures were: in *A*, O_2 2·6 %, N_2 97·4 %; in *B*, O_2 2·6 %, N_2 10·5 %, CO 86·9 %; in *C*, O_2 21 %, N_2 79 %. Curve *C* shows the effect of adding *M*/1000 KCN. Temperature 11°.

It is clear from these experiments that in the succinoxidase system the action of the cyanide is upon some substance which is not inactivated by CO.

These results make it clear that the inhibiting effect of carbon monoxide on the utilisation of oxygen by biological oxidation systems is (like that of cyanide) by no means universal, as Warburg seems to imply, since at least three typical biological oxidation processes are unaffected by it; and, secondly, they show that cyanide and carbon monoxide do not necessarily always inhibit the uptake of oxygen by acting in the same way or on the same substance. If it is assumed that cyanide inhibits the oxidation of succinic acid by inactivating an oxygen-transporting substance, and that carbon monoxide inhibits tissue respiration by acting in the same manner, it follows from these results that different oxygen-transporters must be concerned in the two cases.

SUMMARY.

Carbon monoxide has no inhibitory effect on the aerobic oxidation of aldehyde or hypoxanthine by the milk oxidase, or of succinic acid by the succinoxidase of muscle. Its action thus differs from that of cyanide, which strongly inhibits the latter reaction.

I should like to express my sincere thanks to Sir F. G. Hopkins for his interest in this work, and also to Dr Keilin for suggestions and for the use of apparatus. I am also much indebted to Mr Tate for preparing the CO and for assistance with the manipulations.

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CLXII. THE EQUATION OF ALCOHOLIC FERMENTATION.

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THE fact that the hexosediphosphate which is produced by the action of yeast preparations on a mixture of a hexose and a phosphate is accompanied by a monophosphate renders it necessary to reconsider the equation originally proposed by Harden and Young [1906] to represent the reaction:

$$2C_6H_{12}O_6 + 2Na_2HPO_4 = C_6H_{10}O_4(PO_4Na_2)_2 + 2CO_2 + 2C_2H_6O + 2H_2O.$$

This equation was originally established on the basis of the ratio of carbon dioxide evolved to the phosphate which went into organic combination as experimentally observed and of the composition of the hexosephosphate produced. It was further confirmed by experiments in which the ratio was determined of the sugar added in presence of excess of phosphate to the carbon dioxide produced.

It is however obvious that if some of the phosphate and some of the sugar form a compound of a different composition (a hexosemonophosphate) experiments of the kind just indicated are not sufficient to afford a rigorous proof of the equation.

It was therefore decided to make a more detailed study of the reaction and to endeavour to determine the exact proportions of the hexosemonophosphate and hexosediphosphate produced, as well as the amount of carbon dioxide evolved.

Determination of the hexosemonophosphate and hexosediphosphate produced in the reaction.

The following method has been developed from a suggestion for which we are indebted to Dr R. Robison.

The fermentations are stopped by adding enough 25 % trichloroacetic acid to raise the concentration to 3·5 %. The liquid is filtered and the amount of free and total phosphorus in the filtrate is estimated by Neumann's method. To the rest of the filtrate is added excess of barium acetate and enough hot baryta to render the liquid just pink to phenolphthalein. Enough CO_2 is then added just to remove the pink colour. The liquid is allowed to stand in the cold room for 18 hours and filtered. The free and total phosphorus are then estimated in the filtrate.

It is assumed that in this treatment the large concentration of Ba ions present decreases the solubility of the barium hexosediphosphate to such an extent that this salt is almost completely precipitated along with the free phosphate present, whilst the readily soluble barium hexosemonophosphate is left in solution.

The amount of the latter is calculated from the combined phosphorus found in the liquid. The amount of diphosphate is estimated from the total combined phosphorus less that remaining in solution after precipitation.

Attention must be drawn to two possible errors, which affect the results in opposite directions. (1) If too much baryta is added, some of the monophosphate may be precipitated in the form ot a basic salt. (2) The presence of a considerable concentration of monophosphate may enhance the solubility of barium hexosediphosphate. The extent to which these occur cannot readily be gauged.

In the earlier experiments the total free and combined phosphorus was precipitated by adding 50 % alcohol in conjunction with the barium acetate and baryta. The precipitate so formed was washed with 60 % alcohol to remove sugar, and its content of free and total phosphorus and copper-reducing power determined. From these data by means of a simultaneous equation the amounts of hexosemonophosphate and hexosediphosphate were then calculated. The results were unreliable owing to the presence of barium compounds of glucose or fructose in the washed precipitate.

Attempts were also made to precipitate the hexosediphosphate alone from the fermented liquid by the addition of brucine, but the results obtained did not agree among themselves, probably owing to the slow precipitation of free phosphate by brucine.

Determination of the CO_2 produced in the reaction.

The total volume of CO_2 produced was determined in the usual manner.

Part of this is due to the conversion of phosphoric into the stronger hexosephosphoric acids, the potassium salts of which are hydrolysed to a smaller extent in solution than the phosphates and therefore take up less CO_2 in the form of bicarbonate.

To estimate this part of the gas parallel experiments are made in each of which the same amount of phosphate is added.

To one, excess of trichloroacetic acid is added immediately after the addition of phosphate and the evolved gas measured. This gives the CO_2 combined in the form of bicarbonate at the commencement of the observation.

To the second, the same quantity of trichloroacetic acid is added after all the free phosphate has been combined and the evolved gas measured. This gives the residual bicarbonate. The difference between the volumes of gas in the two cases is taken to represent gas evolved owing to the difference in properties of the hexosephosphates and phosphates.

To reduce this correction to a minimum a mixture of four volumes of

0·6 *M* KH_2PO_4 and one volume of 0·6 *M* K_2HPO_4 was used whenever possible. This mixture can be successfully employed with yeast-juice[1]. In parallel experiments with the same sample of juice the addition of 10 cc. of 0·6 *M* K_2HPO_4 or 10 cc. of the 0·6 *M* phosphate mixture produces the same evolution of CO_2 within the limits of experimental error, when all corrections have been made. The phosphate mixture was found unsuitable for use with zymin or dried yeast, probably on account of its acidity (p_H 6·4).

Another deduction remains to be made from the total volume of gas produced. It has been assumed in past experiments, that, throughout the whole period, during which the added phosphate is undergoing esterification, a steady rate of evolution of CO_2 is maintained by the constant rate of supply of free phosphate by the action of the hexosephosphatase of the yeast preparation on the hexosediphosphate present in the liquid, as shown by the equation

$$C_6H_{10}O_4(PO_4Na_2)_2 + 2H_2O = C_6H_{12}O_6 + 2Na_2HPO_4.$$

This amount cannot be ascribed to the phosphorus added and represents the amount of gas which would have been evolved in the absence of added phosphate.

Some uncertainty attaches to this calculation. The possibility exists that, in the very varied range of concentrations both of phosphate and hexosephosphate which occur in an experiment, the rate of action of the hexosephosphatase may be modified. This is obviously the case when a high concentration of phosphate is employed, since the rate of fermentation is at first considerably depressed and only regains its original value after a more or less prolonged period of induction. In Exps. 66 and 68 (Table I), for example, in which 15 and 20 cc. of 0·6 *M* K_2HPO_4 were used, on the addition of the phosphate the rates fell from 4·8 to 2·4 and 1·8 cc. per 5 minutes and did not regain their original value for 50 and 100 minutes respectively.

Hence, in determining the equivalent between hexosephosphate formed and carbon dioxide evolved only such quantities of phosphate can be used as cause no preliminary depression in the rate of fermentation.

Again, at the close of the experiment, when the concentration of hexosephosphate has become much larger than its original value, the steady rate of fermentation is frequently somewhat greater than before the addition of phosphate.

The complete conversion of the quantities of phosphate which have been used in these experiments requires from 1·5 to 3 hours. The normal rate of fermentation for 25 cc. yeast-juice is about 2–3 cc. per 5 minutes; for 4 g. zymin, 4·5 to 5 cc. and the volume equivalent to the phosphate added is 67·2 cc. for 5 cc. of 0·6 *M* phosphate.

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[1] The statement made by Harden [1923] that the correction is negligible "when a mixture of five molecular proportions of K_2HPO_4 with one of KH_2PO_4 is used" should read "when a mixture of five molecular proportions of KH_2PO_4 and one of K_2HPO_4 is used."

Hence an error of 0·5 cc. in the estimated "normal" rate per 5 minutes in an experiment lasting 1·5 hours involves an error of 9 cc. or 13 %.

The addition of phosphate does not produce so great a relative increase in the rate of fermentation given by zymin as it does in the case of yeast-juice. As the result of this the correction in the case of zymin (see table) is very much higher—and correspondingly less accurate—than in that of yeast-juice.

EXPERIMENTAL METHOD AND RESULTS.

Experiments were carried out both with yeast-juice and zymin (yeast dehydrated with acetone). 25 cc. of yeast-juice were employed in each case + 3 g. fructose or glucose + 0·3 cc. toluene; in the case of zymin 4 g. were used + 3 g. fructose or glucose + 30 cc. H_2O + 0·3 cc. toluene.

In each case three separate quantities of the mixture were incubated at 30° until the rate of evolution of CO_2 became constant. The desired amount of phosphate solution previously saturated with CO_2 at 30° was then added to two of them, which were then treated as already described.

The third was employed to obtain a determination of the hexosemonophosphate and hexosediphosphate in the fermenting mixture at the commencement of the experiment. For this purpose trichloroacetic acid was added in order to stop the fermentation and then the same amount of phosphate as was added to the other two. In the filtrate from this hexosemonophosphate and hexosediphosphate were determined. As a check the same process was sometimes carried out with the yeast-juice or zymin without the addition of phosphate.

In calculating the results the amounts of monophosphate and diphosphate found at the commencement of the experiment were subtracted from those found at the end, the differences being regarded as the amounts of the two esters formed during the experiment. The results are contained in Table I.

Table I.

			P		CO_2 cc at N T P										
Exp. no	Material used	Sugar	Mixture used 0·6 *M* phosphate	mg added	Total evolved	Correction for combined CO_2	Correction for hydrolysis	Corrected vol	Duration no of 5 min. periods	Mg. P as hexosediphosphate	Mg. P as hexosemonophosphate	CO_2 / Combined P	CO_2/ Diphosphate	CO_2/ Monophosphate	Diphosphate/ Monophosphate
46	Yeast-juice	Fructose	10 cc. acid mixture	181·6	155·4	7·6	31·5	116·3	11	133·2	53 4	0·87	2·43	3·03	1·25
50	"	"	"	181·6	148·7	5 3	23·5	119·9	11	96 7	79 6	0·95	3·45	2·10	0·61
58	"	"	"	181·6	148 1	7·5	29·3	111·6	16	144·2	41·1	0·83	2·14	3·75	1·75
54	Zymin 1	"	10 cc. K_2HPO_4	179·7	215 2	21·0	90·8	103·4	17	128·8	39 8	0·85	2·23	3·62	1·62
56	"	"	"	179·7	234·3	21·9	113 2	99·2	18	123·2	43·1	0·83	2·24	3·21	1·43
60	Zymin 2	"	5 cc. K_2HPO_4	89·85	92·6	10·4	53 9	28·3	23	50 6	35·0	0·16	1·55	1·12	0·72
62	Zymin 5	"	"	89·15	142·2	14·4	69·7	58·1	15	83·2	8·0	0 88	1·93	10·08	5·18
64	"	"	10 cc. K_2HPO_4	178·3	208·9	21·0	106·7	81·3	23	131·0	38·6	0·65	1·68	2·91	1·73
66	"	"	15 cc. K_2HPO_4	267·1	233·1	32·2	uncertain		29	215	44·9	not determined			2·39
68	"	"	20 cc. K_2HPO_4	356·6	305·6	42·6	"		41	301·2	59·6		"		2·55
72	"	Glucose	5 cc. K_2HPO_4	89·15	151	12·1	75·6	63 3	17	64 4	26·8	0·96	2·72	3·27	1·20
74	"	"	"	89·15	146·4	11·8	74 4	60·2	17	73·0	18·2	0·91	2·28	4·57	2·00
75	"	Fructose	"	89·15	136·1	11·8	68 0	56 3	15	81·1	10·1	0 85	1·92	7·69	4·00
76	"	Glucose	7·5 cc. K_2HPO_4	133·7	184·4	16·1	93·6	74·7	23	115·1	19·2	0·90	1·79	5·37	3 00
77	"	"	5 cc. K_2HPO_4	90·75	137·5	12·6	65·2	59·7	15	67·9	22·7	0·91	2·43	3·64	1·50

DISCUSSION.

Ratio of CO_2 to total P esterified.

The fact that the ratio of CO_2/P is invariably below unity seems to indicate quite clearly that some esterification has taken place without evolution of CO_2 in accordance with the requirements of the original equation. As the estimation of the total amount of combined P is susceptible of a high degree of accuracy this conclusion may be regarded as well established. This is in agreement with Meyerhof's view [Meyerhof and Lohmann, 1927] that in alcoholic fermentation a monophosphoric ester is first formed without evolution of CO_2, if it be supposed that some of this still exists in the solution (either in its original supposedly active form or in a stabilised form) when the sample is taken for analysis.

Excluding the two exceptionally low results of Exps. 60 and 64, which are discussed later (p. 1221), the average ratio for the remaining eleven experiments is 0·89, the variations being from 0·83–0·96. If we assume that Harden and Young's equation correctly represents the change which results in evolution of CO_2, this indicates that an average of 11 % of the total phosphorus which has entered into combination has been esterified without evolution of carbon dioxide.

Since moreover, as will be seen later (p. 1222), there is no evidence from these experiments that diphosphate is formed without evolution of CO_2, it is probable that this direct esterification results in the formation of a monophosphate. This process then accounts for a considerable proportion, and in some cases for the whole, of the monophosphate present at the end of the experiment.

Ratio of CO_2 to hexosediphosphate.

Experiments have been made with three different preparations of yeast-juice and with three of zymin. Both glucose and fructose have been used. It will be seen that the results vary considerably, the values for the molecular ratio CO_2/hexosediphosphate extending over a comparatively wide range (1·55–3·45). How far this is due to imperfections in the analytical methods employed and how far to variations in the conditions it is difficult to decide. Duplicate analyses of the same experimental liquid give satisfactorily concordant results. On the other hand a series of three experiments (Nos. 72, 74 and 77) with the same sample of zymin at fortnightly intervals, under what were intended to be precisely similar conditions, gave decidedly different values both for the ratio CO_2/hexosediphosphate (2·72, 2·28 and 2·43) and for diphosphoric ester/monophosphoric ester (1·20, 2·0, 1·5). The barometric pressure was slightly different on these three occasions (751, 757·9 and 753·8 mm.), but whether the resulting small differences in the amount of CO_2 retained by the solution and the p_H of the liquids would affect the result is not known.

The lowest result obtained for this ratio, 1·55, was given by a sample of zymin (Exp. 60) which had been preserved for two years and fermented sugar at a much lower rate than the freshly prepared samples. The duration of the experiment was thus considerably prolonged and the corrections to be applied to the gas volume (see p. 1218) rendered correspondingly uncertain. The same is true of Exps. 64 and 76 in which an excessive amount of phosphate was added and the duration of the experiment was unduly prolonged. Excluding these three experiments, in only one experiment out of the twelve remaining did the monophosphate exceed the diphosphate. This gave the highest value obtained, 3·45, for the ratio CO_2/diphosphate. The remaining experiments, in all of which the diphosphate exceeded the monophosphate, gave ratios varying from 1·92–2·72 (nine experiments), the average being 2·38, whilst the average of the series of ten experiments is 2·26. This average agrees approximately with the value 2 which is required by Harden and Young's equation, but the individual variations are somewhat higher than would be expected, even when regard is taken of the uncertainty of the analytical methods and of the correction applied for hydrolysis to the total volume of CO_2 evolved.

The fact that this ratio, CO_2/diphosphate, is almost without exception above the value, 2, required by the equation might, however, be simply explained by assuming that the CO_2 was produced in accordance with the equation, but that some of the hexosediphosphate was subsequently partially hydrolysed, yielding a monophosphate and inorganic phosphate, the latter of which again entered into reaction.

The monophosphate actually present in the liquid analysed would then be derived from two distinct reactions, (*a*) direct esterification (see p. 1229), (*b*) partial hydrolysis of hexosediphosphate. This idea is quite in accordance with the fact that the monophosphate isolated from yeast-juice is probably a mixture [see Robison, 1922; Meyerhof and Lohmann, 1927]. On the other hand no definite evidence of the production of a monophosphate by hydrolysis of diphosphate by yeast enzymes is at present available, although this readily occurs on hydrolysis by acids [Neuberg, 1918], and Robison [1923] has obtained evidence that it probably also occurs when the diphosphate is hydrolysed by the phosphatases of cartilage and muscle. Quite recently Neuberg and Leibowitz [1927] have actually prepared the monophosphate from the diphosphate by the action of taka-diastase. Further work on this question is in progress.

It must however be remembered that if this be the origin of part of the monophosphate, a corresponding amount of inorganic phosphate must be liberated, which again enters into the reaction, since there is very little free phosphate present at the stage when the samples are taken. The carbonic acid liberated by these secondary reactions:

$$C_6H_{10}O_4\,(PO_4H_2)_2 + H_2O = C_6H_{11}O_5\,(PO_4H_2) + H_3PO_4$$

$$2C_6H_{12}O_6 + 2H_3PO_4 = C_6H_{10}O_4\,(PO_4H_2)_2 + 2CO_2 + 2C_2H_6O + 2H_2O$$

is comprised in that entered in the table under the heading "Correction for hydrolysis" and should, if the idea under discussion is correct, be not less than equivalent to the part of the monophosphate formed by hydrolysis. This is the case in every instance except that of Exp. 50, the exceptionally high ratio of CO_2/diphosphate and the large production of monophosphate in which remain unexplained.

Further light on the subject could probably be obtained by making a series of estimations of the relative amounts of monophosphate and diphosphate present at various stages of the fermentation, and efforts are being made to do this. Experiments of this kind as regards the total esterification have been made by Euler and Johansson [1913] and by Meyerhof and Lohmann [1927], but the distribution of phosphorus between the mono- and di-esters has not yet been ascertained.

The present experiments afford no evidence of the formation of diphosphate either directly or indirectly by esterification of monophosphate without evolution of CO_2 [cf. Meyerhof and Lohmann, 1927].

Ratio of diphosphate to monophosphate.

The ratio diphosphate/monophosphate is highly variable, the values obtained ranging from 0·61 to 5·18.

No definite relation of this value to any of the conditions of the experiment—concentration of phosphate (Exps. 62, 64, 66, 68), nature of sugar (Exps. 72, 74, 76, 77), nature of yeast preparation—can be made out. As already mentioned, in only two cases out of fifteen was the production of monophosphate greater than that of diphosphate, on many occasions (*e.g.* Exps. 62, 75, 76) it was very much less. The average value was 2·06. The factor controlling the relation of these two products has so far escaped detection.

Ratio of carbon dioxide to monophosphate.

The ratio of CO_2/monophosphate, like that of diphosphate/monophosphate is very variable—ranging irregularly from 1·12–10·08, with an average of 4·16. The variations are such as almost to preclude the possibility of the existence of a definite relation between the amounts of CO_2 evolved and monophosphate produced.

SUMMARY.

1. The reaction which occurs when a mixture of a hexose and inorganic phosphate is fermented by yeast-juice or zymin (yeast treated with acetone) has been re-examined and the amounts of carbon dioxide, hexosemonophosphate and hexosediphosphate produced have been determined.

2. The ratio CO_2/total P esterified is on the average 0·9, indicating that, if Harden and Young's equation be taken as correct, about 10 % of the phosphorus is esterified without evolution of CO_2. The product of this esterification is probably a monophosphate.

3. The ratio CO_2/diphosphate is on the average 2·38 but varies considerably in individual cases. The fact that this ratio is almost invariably somewhat greater than the value 2 required by Harden and Young's equation suggests that the diphosphate is originally produced in accordance with the equation, but that a part of it is subsequently partially hydrolysed, with formation of a monophosphate.

4. The ratios CO_2/monophosphate and monophosphate/diphosphate are highly variable and show no definite relations.

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CLXIII. EXPERIMENTS ON BACTERIA IN RELATION TO THE MECHANISM OF ENZYME ACTION.

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INTRODUCTION.

It may be urged that resting or non-proliferating bacteria do not form suitable material for experiments on the mechanism of enzyme action; for the reason that the reactions to which such bacteria give rise may be due, not so much to the enzymes as to the circumstance that they form organised systems capable of effects not brought about by constituent members of the systems.

In fact, it may be urged, a living, though not proliferating, cell may possess some property which is expressed by the reactions we are investigating and which would not be possessed by the cell were it disorganised or dead. That this criticism is groundless is shown by the following evidence.

1. If a suspension of *B. coli* be shaken with toluene or ether, the organism is no longer capable of reproduction on the usual nutrient media and therefore, so far as we can ascertain, is dead. But it can still give rise to a large number of activations, *e.g.* with formic, lactic, and succinic acids, at rates not markedly different from those induced by the normal or untreated organism.

2. The effect of exposing *B. coli* to some abnormal treatment (resulting usually in the death of the organism) is to produce, as exposure is prolonged, a step by step elimination of the various activating mechanisms, as though each mechanism were differently susceptible to the treatment. Now, were the activations of the cell due to the latter acting as an organised whole, we should expect that at some particular point the cell would become disorganised and its activations would cease. Each mechanism would be eliminated at the same time and there would be no step by step degradation. Since this is contrary to what actually occurs we cannot suppose that the activations of the cell depend upon the survival of the latter as an intact organised system.

We have indeed *no* evidence to support a contention that the reactions of the organism are dependent upon its survival as a living unit. It is true that when an organism is killed certain of its activations (usually those of the sugars in the case of *B. coli*) disappear, but the simple interpretation is that the agent responsible for the death of the organism is also responsible for the disappearance of the activating mechanisms--not that the disappearance occurs because the organism has been killed.

There can be no doubt, however, that the power of the cell to proliferate is dependent upon the intactness of (among other things) many of the activating mechanisms with which we have to deal. This is entirely to be anticipated, for we cannot expect a cell to survive, whose cellular or intracellular structures have been so changed, by exposure to abnormal conditions, that they can no longer perform activations (which, on our views, are simply an expression of the nature of these structures).

In a previous communication by one of us [Quastel, 1926] the theory was advanced that the dehydrogenations[1] effected by bacteria are primarily due to polarisations of substrate molecules induced by electric fields which characterise particular centres—the "active centres"--of cellular and intracellular surfaces. The effect of polarisation may be to activate the molecule, *e.g.* it may become a hydrogen donator or acceptor, but since activation necessitates the uptake of a critical amount of energy, it follows that not all molecules which are polarised will become activated. This will depend on the nature and intensity of the polarising field and upon the electrical and chemical nature of the substrate molecules. The mechanism by which a substrate, when activated, becomes a hydrogen acceptor or donator is discussed in earlier papers [Quastel, 1926; Quastel and Wooldridge, 1927] and will not be discussed further here.

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[1] Such dehydrogenations do not refer to reductions brought about by glutathione or substances which are active *in vitro*; they refer primarily to substances which must be activated by the cell before their oxidation can occur.

The view there put forward makes it possible to interpret in a consistent manner such phenomena of biological oxidations as β-oxidation, asymmetric oxidation of the double bond, the α-oxidation of propionic acid, the mode of oxidation of branched chains and so forth. It also indicates how it is possible to regard specificity of behaviour as belonging to the molecules themselves; it being unnecessary to postulate the existence of numerous specific enzymes related to oxidations. Thus one active centre may be able to effect the activation of a number of substrates, the chance of one molecule rather than another being activated depending upon its structure. But specificity of behaviour must depend not only on the chance of the molecule being activated at a particular centre but also on its power of obtaining access to that centre. If the structure of the molecule is such that, although it may become activated by the centre, it cannot reach the centre, or remain there for a sufficient period of time for activation to occur, it is clear that the molecule will not react. Specificity, then, is also a function of the accessibility of a molecule; adsorption and appropriate orientation are factors which help the access of a molecule to a centre.

The "active centre" is a part of, or a property of, cellular and intracellular surface structures; it is inseparable from surface structure, as it is a function of it. Some conception of its properties and mode of formation will be given in Section V. It is not necessary to believe that only surfaces of considerable magnitude are capable of possessing such centres. Any surface structure, however small, may possess such centres; this will depend upon the nature of the surface. This view is discussed more fully later.

If a surface structure be attacked by chemical or by physical means a change in the number and type of the active centres distributed over the surface will take place. The change will be dependent upon the extent to which the surface structure is changed. The centres which are least resistant to "shocks" (though the resistance must vary, we expect, to some extent with the type of "shock") will be the first to disappear as the surface structure is attacked. But it does not follow that an active centre must *entirely* disappear as a result of change. It may form a new centre, according to the nature of the change, with a smaller strength of field, or perhaps a field of quite different nature. As a surface structure is attacked, the general effect, we expect, will be that the least stable active centres are first eliminated; then come those relatively more stable and finally the most stable. Thus there will result a definite sequence of eliminations which will proceed step by step. The sequence will be the same, as a whole, for a large number of methods of treatment of the organism—but that it is unlikely *always* to be so is clear, for individual variations, due to particular effects of an attacking reagent, will on certain occasions make themselves manifest. Thus a certain reagent may react vigorously with a certain constituent group of the active centres, rendering inert the substrates which are made accessible to the centres by means of this group. Yet the elimination of this group may not influence very markedly the activating power of these centres, and so substrates whose accessibility is unaltered are still activated. Such a reagent would be liable to give a different sequence of eliminations from that produced by another reagent.

That the general effect of reagents is to give a sequence of eliminations which remains as a whole the same is illustrated by experiments described in our last paper [Quastel and Wooldridge, 1927][1] where it was noted that a variety of different treatments of *B. coli* gave the same general order of eliminations. Experiments described in Section III will further illustrate this.

The importance of accessibility—*e.g.* adsorption or orientation—of a substrate as a factor in the determination of specificity will be discussed later, but it is as well to point out now that although a molecule may become activated at a centre it may not, in this activated state, undergo the reaction in question until it has passed through a particular phase of internal (electronic) structure or through a particular phase of stability. Various factors, such as orientation or adsorption, may influence the time necessary for the activated molecule to reach this phase. This interval of time is of importance, for it is possible that the molecule may become deactivated by collision before it has time to react. If the substrate molecule, through some property of its own structure or of that of the active centre, is unable to remain in contact with the centre for a sufficiently long period of time, its chance of reacting becomes small, in spite of the fact that the centre may have sufficient power to produce an activation. On the other hand, if there be present in the centre a grouping (or set of groupings) which will hold the molecule for a sufficient period of time reaction will ensue. This grouping is the "active group" of an enzyme. On our views it is simply one of the factors influencing accessibility of a substrate and hence influencing the specific behaviour of the active centre. The centre, *as a whole*, activates; one or more of its groupings is responsible for the access of the substrate. It may be possible to modify or destroy the centre, without necessarily altering the groupings responsible for access, *i.e.* the substrate may still be adsorbed without activation occurring. Or again, it may be possible to eliminate a group responsible for access without reducing the field strength to such an extent that it can no longer activate the molecule. The importance of this point lies in the fact that we should be able to differentiate between two molecules, activated at the same centre but each requiring a different group to make it accessible. By eliminating such a group one substrate will be rendered inert whilst the other molecule, whose access is unaltered, may still be capable of activation by the centre. Thus the centre would have the appearance of being composed of two specific enzymes.

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[1] The references [Quastel, 1926] and [Quastel and Wooldridge, 1927] will, from this point, be represented by the signs [1] and [2] respectively. These papers should be consulted for full details of the experimental technique adopted in this communication.

Specificity, then, is a function of the following factors.

1. Nature of the polarising field at the active centre.
2. Structure of the substrate molecule.
3. Orientation and adsorption of the substrate molecule at the centre; these depend also on the chemical structure of the centre itself.

It will be seen that a centre may easily differ from a neighbouring centre. Each has an individuality of its own—each may, indeed, be regarded as a specific enzyme but with a certain limited range of specificity. The two fundamental ways in which an active centre differs from a truly specific enzyme are:

(*a*) a range of specificity which is limited—characteristic of each centre:

(*b*) the identity of an active centre with a surface structure.

Willstätter [1927] states: "It seems that we must consider an enzyme to be composed of a specifically active group and a colloidal carrier....The colloidal carrier seems to vary somewhat in its nature, but to be necessary for the stability of the active group." This statement can be regarded as an approximation to the view of active centres, it being emphasised once more, however, that on this view activation is due to the centre as a whole, probably to a composite of groups, and not necessarily to a specifically active group. The latter, whilst forming an essential feature of the centre, may be important primarily in securing the access of the substrate to the activating field.

Let us now consider the evidence which leads us to suppose that activations (dehydrogenations) due to bacteria are associated with surface action.

I. Evidence for surface action in the dehydrogenations produced by *B. coli*.

Whilst the evidence from work on enzymes points very clearly to the participation of surface action it is difficult to prove rigidly, by one particular experiment, that in the activations due to bacteria surface activity is fundamentally involved. It is only when the evidence as a whole is taken into account that there seems to be no escape from this conclusion, except by postulating a number of assumptions which are usually highly questionable and for which we have no evidence. By surface activity is meant activity by surfaces, either cellular or intracellular.

Quantitative measurements [1] have already indicated that the main site of reduction of methylene blue by a hydrogen donator is at the cell surface and for reasons given in that paper it was shown that *a site* of activation of hydrogen donators is also at the cell surface.

If a suspension of *B. coli* be shaken with toluene [2] a number of activations (notably those of the sugars and glutamic acid) are eliminated, whilst others (succinic acid, lactic acid, formic acid) are retained. We may try to explain this purely on permeability considerations. The argument would be, presumably, that the effect of the toluene is to act selectively on the cell membrane, rendering it impermeable to the sugars but not to succinic acid, etc. Now this selectivity of action cannot be attributed merely to mechanical blocking for whereas glycerol is made inactive, α-glycerophosphoric acid is as active after as before treatment; again, methylene blue must still be freely permeable for the rates of action due to formic acid, etc., have not changed. We may then suppose that the selectivity of action is due to the formation of a film, at the surface, possessing selective permeabilities, but this cannot be true, for the formation of such a film would not allow methylene blue to penetrate it. Methylene blue does not diffuse, under the conditions of our experiments, from aqueous solution into toluene and we should expect a film of toluene at the cell surface to act like a lipoid layer which, as we know from the work of Loewe [1912], would adsorb but not dissolve the dyestuff, *i.e.* the latter would not penetrate into the cell.

We may suppose finally that there is a specific entrance into the cell for the sugars, glycerol and glutamic acid and another one for succinic acid, lactic acid, methylene blue, etc., and that toluene blocks the specific entrance for the sugars and not the other entrances. Now from the results of other experiments we can demonstrate differences between succinic acid and lactic acid, between glycerol and glucose, etc., so that if the interpretation of our results is simply to be that of specific attacks on pores specific for substrates we would have to assume a specific pore for almost every substrate—a conclusion which seems to be entirely out of the question.

Since the problem does not find a satisfactory solution simply in the consideration of changes in permeability, we may take the view that the toluene penetrates the cell and there affects certain specific enzymes, *i.e.* those related to the sugars, etc. But ether, benzene, chloroform, propyl alcohol, all have extremely similar effects to toluene, and since it is scarcely conceivable that such substances as these form definite chemical compounds with the enzymes, it is reasonable to suppose that they would act in the manner we would anticipate from such capillary active substances. We expect that they would form films upon the specific enzymes, rendering the latter inaccessible to methylene blue or to the substrates or to both. This clearly indicates, as we would naturally expect from other work on enzymes, that the specific enzymes for the dehydrogenations are associated with surfaces of some description. This does not apply only to the sugars, for experiments with propyl alcohol indicate that the other specific enzymes—those for succinic acid, lactic acid, etc.—are equally associated with surface action.

If the toluene does *not* enter the cell, thus affecting certain enzymes therein, we must imagine that it forms a film upon a particular patch or series of patches on the cell surface, these patches being particularly associated with the activations of the sugars, etc. If these patches were not so associated there seems to be no reason why the sugars and methylene blue should not enter the cell at parts of the surface not affected by toluene and so react with enzymes in the cell.

Were we to ascribe the effects of treatment of *B. coli* by nitrites [2] or by high concentrations of salts [2] or by the halogens or by allyl alcohol or by sodium acrylate (Tables I and II) to changes in permeability of the cell membrane alone, we should be placed in the difficult situation of having to account for the fact that whilst a variety of donators are prevented from entering the cell, methylene blue appears to be freely permeable always. For if it were not so, formic acid could not react at its normal rate when many of the other donators have been rendered inert. Again, it seems inconceivable that such a variety of attacks should change the permeabilities of the substrates in almost exactly the same order. The simple interpretation of these facts is that the main site of reduction of methylene blue is at the cell surface, supporting the conclusion drawn from quantitative evidence.

It seems, therefore, from various lines of evidence that surface activity is intimately associated with the activations of many hydrogen donators. The evidence is based upon experiments carried out with methylene blue, and *with this dye-stuff* it appears that the outer cell surface of *B. coli* is the main site of reduction; hence this surface must constitute *one* important site of the activation of hydrogen donators. Methylene blue has been valuable in demonstrating this fact and hence in indicating clearly the importance of surface action. But it would be absurd to consider that because, with methylene blue, the outer cell surface is the main site of reduction, activations cannot be performed within the cell. The facts that mechanical disintegration of bacteria appears to have a destructive action on certain activating mechanisms [1] and that lysis of the organism by bacteriophage ultimately removes the power of the cell to reduce methylene blue [Shwartzman, 1927; Gozony and Surányi, 1925] support our conclusion that the cell surface is a site of activations, but we cannot expect that the breakdown of the cell surface will necessarily involve the destruction of all intracellular surfaces and the elimination therefore of all activating power. Our thesis has been to show that surface activity, whether it be cellular or intracellular, is fundamentally connected with the activations we are investigating and it should therefore be borne in mind that when reference is made to surface activity, the term surface includes both cellular and intracellular surfaces.

Let us now consider briefly the phenomenon of activation and the conception of active centres.

II. ACTIVATION.

It has been necessary for several reasons to consider that in homogeneous reactions a molecule cannot undergo transformation and dissociation unless it possess a certain minimum internal energy. "By internal energy is meant all the energy it possesses over and above what it would possess at very low temperatures except energy of translation, which, by the principle of relativity, can have nothing to do with the possibility of an internal change. Any molecule containing an amount of energy equal to or greater than a critical amount may be called an activated molecule. It may not be in a position to undergo the particular transformation we have in mind for this may depend upon the orientation of the several parts of the molecule,. upon the location of the energy within the molecule and upon other factors of a similar nature" [Lewis and Smith, 1925].

Two types of chemical union are recognised: the electrostatic attraction between oppositely charged ions, this is a polar linkage; and the attraction which is produced through the sharing of an electron by two atomic nuclei, this is a non-polar linkage. We suppose that for the dissolution or formation of a non-polar linkage there must be primarily a shift of the electrons from their original orbits. This necessitates the uptake of energy, the process being termed activation. A critical amount of energy of activation must be taken up by a molecule for reaction to be possible.

Ions do not require activation in ionic reactions with which we are familiar, for such reactions consist simply of rearrangements of polar linkages. There is no necessity, for instance, to postulate activation of hydrogen or hydroxyl ions.

At present two fundamental methods of activation are recognised, collision and radiation. It is unnecessary, for the purposes of this paper, to go into the processes in detail, but it is important to note the following points.

1. An activated molecule may be deactivated by collision.

2. An activated molecule may not react until a particular phase of the internal structure of the molecule has been reached [Lindemann, 1922; Thomson, 1927]. We may expect a definite time interval between time of activation and time of transformation, the interval depending on the way in which activation has been effected.

Active centres.

Much of the work on surface catalysis carried out within recent years[1] has gone to show that catalytic activity occurs at particular areas of the surface and that it varies in nature according to the nature of these areas. The areas, or active centres, constitute as a whole the active surface of the catalyst.

Adsorbed molecules at an active surface are regarded as in a distorted condition, or in a state of strain, the amount of distortion depending upon the molecular structure.

It has been shown that if a reaction catalysed by a surface occurs between two reactants both must be adsorbed and activated [see Hinshelwood, 1926].

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[1] The subject is reviewed in the *Annual Reports of the Chemical Society*, **22**, 17. Owing to the existence of this report we refrain from quoting the literature concerned with surface catalysis and active centres. But fully to understand this section, the report should be read by those unacquainted with the literature.

The value of adsorption, of some description, at an active centre is obvious when it is considered that we cannot expect activation at the centre, in general, to be instantaneous. When it is considered, too, that deactivation by collision may occur it is sufficiently patent that appropriate orientation of a substrate molecule (which may lessen the time for activation to occur) and adsorption (which may give time for the activation) are factors of utmost importance in the consideration of the specificity of behaviour of active centres.

It seems to be fully established that the active areas occupy only a small fraction of the surface and that they vary in capacity both to adsorb reactants and to promote reaction.

Taylor [1925] conceives that the activity of metal surfaces is due to incomplete surface crystallisations, so that there are produced occasional groups of atoms associated with high energy and chemical unsaturation relative to the atoms in the regular crystal lattices. [In this connection see also Kistiakowsky, Flosdorf and Taylor, 1927.]

In this paper we shall adopt the view put forward previously by one of us [1] that the activations at biological surfaces occur at the active centres of these surfaces, the mechanism of activation being a polarisation of the substrate due to electric fields at the centres. The specificity of action at the centre depends upon the accessibility of the substrate to the centres, as well as upon the electrical nature of the substrate and the nature of the polarising field.

We are aware that a purely "chemical" view of activations is possible but we find such a view inadequate to interpret our results and unsatisfactory in its inability to give us a rational picture of the mechanism whereby activations are effected. Our own view can be regarded as quite definitely chemical, yet it has the considerable advantage that one is enabled to perceive more clearly the extent to which various factors can operate upon specificity of action, than by a view which calls for a new enzyme related to, and for the formation of a new unstable intermediate compound with, every new substrate which is found to be activated.

We will now enquire into some of the properties of the active centres concerned with the dehydrogenations effected by bacteria.

III. BIOLOGICAL ACTIVE CENTRES.

Treatment with the most diverse reagents has the effect of eliminating the activating mechanisms of *B. coli*. This leads to the conclusion that different groupings may be involved in the activation of substrates and since any one enzyme, or active centre, can be eliminated by any of the treatments, if the latter be carried out for a sufficient period of time, we must conclude that an active centre is made up of a number of groupings.

Or, if we take the attitude that the effect of various reagents is not to attack specifically certain groups in the active centres but to alter surface structure as a whole, so that the active centres are changed or eliminated, we must still conclude that such centres are of a somewhat complex nature and not composed simply of one grouping, as, for instance, the amino- or hydroxyl-group.

It is an interesting fact that approximately the same order of eliminations occurs on submitting *B. coli* to the action of

(*a*) varying concentrations of different salts (Table II);
(*b*) bromine and iodine (Table I);
(*c*) formaldehyde (Table I);
(*d*) allyl alcohol and acrylic acid (Table I).

The action of salts on B. coli.

An examination of Table II, which gives a summary of the experimental results, will show how the same order of elimination of the substrates occurs with all the salts investigated. There are several points of interest arising out of these results.

(*a*) The reduction time for formates appears to keep almost constant in spite of large changes in the reduction times for succinate or lactate or glucose.

(*b*) The iodate ion is more effective than the chlorate ion, and the barium ion far more than the calcium, strontium or magnesium ion in accomplishing inactivations.

The fluoride ion is far more effective than the other halogen ions in attacking, particularly, the glucose-activating mechanism.

(*c*) Relatively low concentrations of salts, with the exception of barium chloride and potassium iodate, do not affect the organism. As concentration is increased the activating mechanisms are selectively eliminated, the bivalent calcium, barium and magnesium ions being more effective than the univalent potassium and sodium ions. The chlorate, bromate and iodate ions are also much more powerful in action than the chloride, bromide and iodide.

The action of bromine and iodine on B. coli.

Very small quantities of these halogens are required to effect the destruction of activating mechanisms of *B. coli*. As shown in Table I, the least resistant are those for glycerol, glutamic acid and succinic acid. Then come those for the sugars, then those for α-hydroxybutyric acid and lactic acid and finally, the most resistant, those for formic, acetic and butyric acids.

The action of formaldehyde and acetaldehyde on B. coli.

Both aldehydes have a powerful action on the activations of *B. coli*, formaldehyde at lower concentrations than acetaldehyde (Table I). The order of elimination is similar to that noted with the halogens, etc. (see Table III), but the sugar-activating mechanisms appear to be rather less resistant.

Table I.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue solution, 1 cc. of the suspension of organism, and the donator, the volume being made up to 7 cc. with physiological saline solution. Reductions were carried out *in vacuo* at 45°. All substances under investigation were brought to p_H 7·4 by sodium hydroxide or hydrochloric acid. Controls with all organisms, before treatment, showed no reduction within 2 hours. The reduction times are given in minutes. The sign ∞ indicates that reduction does not occur within 3 hours. The treatment to which the organism is submitted is described at the head of each column. The organism is always well washed with saline after treatment and made up to its original bulk before use. + indicates that activation occurs, a zero sign that it does not.

	H.CHO 0·2 % 1 hr. p_H 7·4 37°	CH_3.CHO 2 % 1 hr. p_H 7·4 37°	Allyl alcohol 5 % 1 hr. p_H 7·4 37°	Acrylic acid 4 % 1 hr. p_H 7·4 37°	I_2 0·0025 % 1 hr. p_H 7·4 37°	I_2 0·005 % 1 hr. p_H 7·4 37°	Br_2 0·005 % 1 hr. p_H 7·4 37°	Br_2 0·01 % 1 hr. p_H 7·4 37°	Aniline 1 % 1 hr. p_H 7·4 37°	$KMnO_4$ 0·001 % 1 hr. p_H 7·4 37°	$KMnO_4$ 0·01 % 1 hr. p_H 7·4 37°	KCN 1% 1 hr. p_H 7·4 37°	H_2O_2 5 % of 20 vol. ½ hr. p_H 7·4 37°	Average control with organism before treatment
Succinic acid *M*/140	∞	89·5	∞	∞	122	∞	67	∞	37	92	202	49	203	13·5
Lactic acid *M*/140	16·7	32	75	52	19	180	22	140	18·7	29	15	28	50	8
Formic acid *M*/140	5	4·7	3·3	14·5	5	8	6	11	11	180	∞	∞	∞	4
Acetic acid *M*/14	8·7	6·7	4·5	29	6	11	7	16	13	180	∞	∞	∞	5·5
Butyric acid *M*/14	7·5	7·3	6·3	43	7·5	12·5	8·5	13	10	∞	–	∞	∞	7
α-Hydroxybutyric acid *M*/70	40	63·5	173	112	30	∞	30	∞	56	47	39·5	44	135	13·5
α-Glycerophosphate *M*/14	8	8·5	∞	191	6	∞	10	∞	14	150	∞	∞	∞	5·5
Glycerol *M*/7	∞	∞	∞	∞	42	∞	44	∞	∞	77	–	∞	∞	18
Glutamic acid *M*/140	∞	∞	∞	∞	62	∞	114	∞	∞	∞	∞	∞	∞	20
Glucose *M*/140	∞	36·5	∞	∞	13·5	∞	22	∞	∞	27·5	∞	15	∞	8
Fructose *M*/140	78	29	∞	∞	13	∞	20·5	∞	∞	25	–	10·5	∞	6
Mannose *M*/140	∞	44·7	∞	∞	14	∞	31	∞	∞	38	–	19·5	–	7·2
Galactose *M*/140	∞	43·3	∞	∞	13·5	∞	24	∞	∞	43	–	20·5	–	6·5
Mannitol *M*/140	∞	∞	∞	∞	20	∞	38	∞	∞	54	–	27	–	13
Sorbitol *M*/140	∞	∞	∞	∞	–	∞	41	∞	∞	51	–	27·5	–	12
Fumaric acid *M*/70	0	+	0	0	+	0	+	0	+	+	–	+	(+)	+
Na nitrate *M*/140	+	+	+	+	+	+	+	+	+	+	–	+	0	+
K chlorate *M*/140	+	+	+	+	+	+	+	+	+	+	–	+	0	+
Growth on agar from one loopful of treated organism after 18 hrs. at 37°	A few discrete colonies	Slight	None		Good	Very slight	Good	Very slight	None	Moderate	None	Prolific	Very slight	Prolific

Table II.

Reduction times of 1 cc. 1/5000 methylene blue in presence of donators (succinic acid, etc.) by *B. coli* submitted to the action of various salts at various concentrations at p_H 7·4. Organism well washed after each treatment. Conditions as in Table I.

Treatment *coli* 1	t_{succinic}	t_{lactic}	t_{formic}	t_{glucose}	Treatment *coli* 2	t_{succinic}	t_{lactic}	t_{formic}	t_{glucose}
M/5 NaCl	9′	6·25′	3·5′	4·25′	*M*/2 NaCl	16·3′	11′	3′	56′
M/5 KCl	12·5	9·25	4	7	*M*/2 KCl	20	11	3	55
M/5 KBr	12	7·5	4	5·3	*M*/2 KBr	14·5	10·3	3	11
M/5 KI	17	11·75	4·25	9	*M*/2 KI	24·5	13·3	3·3	38·5
M/5 NaF	29·5	18	3·25	24·5	*M*/2 NaF	21	16	3·5	∞
M/5 $KClO_3$	19·5	10·75	4·25	10·5	*M*/2 $KClO_3$	160	27	3	∞
M/5 $NaBrO_3$	22·5	10·5	4	14 25	*M*/2 $NaBrO_3$	150	18	3·7	∞
M/5 KIO_3	∞	35·5	4·5	∞	*M*/2 KIO_3	∞	∞	5·3	∞
M/5 $NaNO_3$	13·5	17·75	4	7·25	*M*/2 $NaNO_3$	23·5	13	3	∞
M/5 $NaNO_2$	30	18	4·5	15	*M*/2 $NaNO_2$	51	19	5·5	∞
M/5 Na_2SO_4	11·5	7	3	6	*M*/2 Na_2SO_4	15·5	13·7	3·5	∞
M/5 $(NH_4)_2SO_4$	11	7·3	3·3	5·7	*M*/2 Na citrate	15	13·7	3·7	∞
M/5 $CaCl_2$	25	11·7	4·7	105	*M*/2 $CaCl_2$	52	37	11	∞
M/5 $SrCl_2$	27	10·3	4·5	11·7	*M*/2 $SrCl_2$	67	26	5·3	43
M/5 $BaCl_2$	∞	∞	165	∞	*M*/50 $BaCl_2$	15	11	4·3	7·5
M/5 $MgCl_2$	36	16	5·5	18	*M*/2 $MgCl_2$	100	31	6·3	∞
Control	7·5	6	4	5	Control	16·5	11	3 3	6·7
M/1 NaCl	18	12·5	3·7	∞	Dist. water for 1 hour	24·5	12	3·3	9·5
M/1 KCl	29	14	4	28					
M/1 NH_4Cl	28	13	4	∞					

Table III.

Showing the general order of eliminations produced by various treatments which are described horizontally. The least stable mechanisms are eliminated first. When two or more mechanisms are eliminated together they are given the same number, and that which is next eliminated is given the corresponding number allowing for those which were previously eliminated.

	Glycerol	Glutamic acid	The sugars	Succinic acid	Fumaric acid	α Hydroxybutyric acid	Lactic acid	α-Glycerophosphoric acid	Formic acid	Acetic acid	Sodium nitrate
Increase of temperature	1	2	3	6	6	4	5	9	9	9	8
Increase of acidity	1	1	6	3	3	5	6	9	10	10	6
Increase of alkalinity	1	2	3	4	.	5	9	5	5	5	.
Hypertonic NaCl	1	2	3	6	.	4	5	7	8	8	.
$NaNO_2$	4	3	6	1	1	4	8	7	9	9	.
Acetone	1	1	1	5	.	4	6	6	6	6	.
Benzene	1	1	1	5	.	4	6	6	6	6	.
Toluene	1	1	1	5	.	4	6	6	6	6	.
Ether	1	1	1	5	.	4	6	6	6	6	.
Phenol	1	1	1	5	.	4	6	6	6	6	.
Chloroform	1	1	1	5	.	4	9	8	6	7	.
Propyl alcohol	1	1	1	5	5	4	6	6	6	6	.
Cyclohexanol	1	1	1	1	1	1	1	8	8	8	.
Aniline	1	1	1	5	.	4	5	7	7	7	.
Methylene blue	1	1	1	5	.	1	6	7	7	7	.
Formaldehyde	1	1	1	1	1	6	7	8	8	8	.
Acetaldehyde	1	2	3	3	.	5	6	7	7	7	.
Allyl alcohol	1	1	1	1	1	7	8	1	9	9	.
Acrylic acid	2	1	3	3	3	7	8	6	9	10	.
Iodine	1	1	5	3	3	5	8	7	9	9	.
Bromine	2	1	5	3	3	6	8	6	9	9	.
KCN	3	4	6	6	6	9	10	5	1	1	.
$KMnO_4$	3	3	6	7	.	8	9	5	1	1	
H_2O_2	1	1	1	8	8	10	11	7	4	4	4

The action of allyl alcohol and acrylic acid on B. coli.

Both these substances have very powerful effects (Table I). As usually observed, the mechanisms for lactic acid and the lower fatty acids are the most resistant to attack.

The order of elimination of activating mechanisms of B. coli.

The order of elimination noted above is similar to that already found on exposure of *B. coli* to nitrites at relatively low p_H or to different hydrogen ion concentrations [2]. See also Table III which gives a complete summary of these results on eliminations.

It would be natural to suspect that the same *order* of eliminations in such a variety of treatments is consequent upon the operation of some common factor underlying all the eliminations. This may be, as we have suggested elsewhere, a general change in the structure of the surface with the result that the most unstable centres disappear first, then those of greater stability and so on. But this view, though accounting for the same order of disappearances, cannot be entirely adequate. It does not take into account specific actions on the centres themselves, which by influencing one or more groups at the centre, may remove accessibility of certain substrates without necessarily affecting, in a marked degree, the activating powers of the centre. Were such groupings affected there would probably be a departure from the usual order of eliminations.

Such a departure from the usual order is noted particularly in the effects of exposing *B. coli* to dilute solutions of potassium permanganate or to relatively strong solutions of potassium cyanide.

The action of potassium permanganate, potassium cyanide and hydrogen peroxide on B. coli.

Here the mechanisms for formic acid and acetic acid, which are usually the most resistant to attack, are among the least resistant (see Table I). Some particular grouping, very sensitive to attack with $KMnO_4$ or KCN, would seem to be particularly associated with formic acid or acetic acid. A somewhat similar effect is produced by treating the organism with H_2O_2[1] (Table I). It may be an aldehyde or carbonyl group, but treatments with sulphites, phenylhydrazine, semicarbazide or aminoguanidine (Table IV) do not exhibit this anomalous behaviour of permanganate or cyanide.

Dilute concentrations of potassium cyanide are almost without action on *B. coli* and relatively strong solutions must be used to exhibit the eliminating action. Potassium permanganate solution, on the other hand, must be used in extremely dilute solution to show its selective effects; relatively strong solutions simply eliminate the entire activating action.

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[1] The effect of H_2O_2 appears to vary somewhat with the strain of the organism, a fact which may be explained by variation in catalase content.

Table IV.

Conditions as in Table I.

Treatment	$t_{glucose}$	$t_{succinic}$	t_{lactic}	t_{formic}
Control (normal organism)	5	7·5	6	4
10 % NaCl (control), 1 hr., p_H 7·4	∞	15·5	11·5	3 7
5 % Semicarbazide, 1 hr., p_H 7·4	12	10	6	3
10 % Semicarbazide, 1 hr., p_H 7·4	∞	24	19	4·7
5 % Semicarbazide, 17 hrs, p_H 6·0	∞	∞	60	5·5
10 % NH_2OH, 1 hr., p_H 7·4	∞	∞	30	5 2
5 % NH_2OH, 17 hrs., p_H 7·4	∞	136	34	9
10 % Na_2SO_3, 1 hr., p_H 6·0	36	21	12	4·7
5 % Na_2SO_3, 17 hrs., p_H 6·0	∞	∞	31	8·7
1 % Phenylhydrazine, 17 hrs., p_H 6·0	∞	∞	50	∞
1 % Phenylhydrazine, 1 hr., p_H 7·4	>60	>60	8	>60
1 % Aniline, 1 hr., p_H 7·4	∞	63	33	6
2 % Aminoguanidine, 1 hr., p_H 7·4	32	33	19 5	4·2

Reversibility.

The action of cysteine and sodium hydrosulphite. So far as we can ascertain at present, the majority of the eliminations effected by the various treatments *B. coli* is made to undergo, are irreversible. Yet it may be that the correct experimental conditions to effect regeneration of the activating mechanisms, apparently irreversibly eliminated, have not yet been obtained and it would be unwise at present to conclude definitely that true irreversibility of action occurs. We can, however, show that some of the mechanisms attacked by potassium permanganate and particularly by potassium cyanide are capable of being regenerated. Exposure of KCN-treated *B. coli* to sodium hydrosulphite solution or cysteine (see Table V) brings about a marked recovery in the time of reduction due to formic and acetic acids. A slight recovery has been observed with the $KMnO_4$-treated organism. As noted also in our previous paper [2] the formic acid or acetic acid mechanism after treatment of *B. coli* with nitrites can be partially regenerated by subsequent treatment with sodium hydrosulphite. Such a recovery does not occur with the succinic acid or lactic acid mechanism.

Table V.

Conditions as in Table I.

Col. A. Reduction times due to *B. coli* treated with 1 % KCN soln. for 1 hr. at p_H 7·4 and 37° and finally well washed.

Col. B. Reduction times due to KCN-treated *B. coli* which has been suspended in 2 % cysteine soln. for 1 hr. at p_H 7·4 and 37° and finally well washed.

Col. C. Reduction times due to KCN-treated *B. coli* which has been suspended in 5 % sodium hydrosulphite soln. for 1 hr. and finally well washed.

Donator	A	B	C
M/28 Succinic acid	13·2	11·7	80
M/28 Lactic acid	6·2	7·2	16·5
M/28 Formic acid	60	9·5	23
M/7 Acetic acid	45	7·7	22
M/14 Glutamic acid	18·5	15·7	60
M/28 a-Hydroxybutyric acid	11·2	12	29
M/28 a-Glycerophosphoric acid	17	11·7	25·5
M/28 Glucose	5·7	6·5	17·5

The action of copper sulphate solution on B. coli *and the reversing effect of* H_2S. Exposure of *B. coli* to dilute solutions of copper sulphate (see Table VI) with subsequent thorough washing, by centrifuging, results in the elimination of activating mechanisms of the organisms. If H_2S be passed through a suspension fo the copper-treated organism and this again well washed, the organism, which is coloured brown, is now capable of activating its substrates. The phenomenon is similar to that described by Myrbäck [1926] in the case of invertase inactivated by silver. It was thought that possibly the copper would protect the activating mechanisms from the action of toluene, aniline or phenol or methylene blue but, as the results in Table VI indicate, no such protective action occurs. It is particularly interesting in this connection that H_2S not only regenerates the activating mechanisms of *B. coli* which have been eliminated by treatment with copper, but it causes a considerable number of the treated cells which are apparently dead to become capable of proliferation. Most cells of *B. coli* are incapable of proliferation after treatment with dilute copper sulphate solution and subsequent thorough washing, but if H_2S be passed through a suspension of such treated cells, many of them now become capable of growing. We have never failed to obtain this result, though if treatment with copper has been too prolonged it is difficult to obtain regeneration of many cells by H_2S. Typical figures are shown in Table VII.

Table VI.

Reduction times in minutes with *B. coli*. ∞ = not reduced in 4 hours.

Conditions as in Table I.

			After exposing copper-treated organism to				
Donator	Normal	After treatment with $CuSO_4$ soln. (1/1000)	H_2S	toluene and finally to H_2S	1 % aniline HCl (p_H 7·4) and then to H_2S	1 % phenol (p_H 7·4) and then to H_2S	methylene blue 1/10,000 soln. and then to H_2S
Succinic acid	13	∞	164	∞	∞	∞	∞
Lactic acid	7	∞	21	20	40	134	117
Formic acid	3·5	∞	4	4	8	9·2	13
Glucose	6	∞	9	∞	∞	∞	∞

Table VII.

To 10 cc. of a suspension of *B. coli* were added 2 drops 10 % $CuSO_4$ soln. This was allowed to stand at room temperature for ¼ to ½ hr., centrifuged and the deposit suspended in 10 cc. sterile saline. A drop of this was spread over an agar plate. The suspension was then submitted to a stream of H_2S for 30′ at room temperature and a drop again spread over agar.

Exp.	Colonies obtained from one drop of suspension after exposure to copper	Colonies obtained from one drop after passing H_2S through the suspension of copper-treated organism
1	80	800
2	59	Prolific >3000

The nature of the active centres.

It seems, from the general results on treatment of *B. coli*, that we must regard an active centre as made up of a number of groupings each of which plays its part in determining the access of a substrate to the centre. If the surface structure as a whole is altered by a number of reagents we expect each reagent to give the same sequence of eliminations. Reagents of this class, which give the same order of eliminations, modify the fields (at the active centres) or affect the stability of the centres by a general change in the entire surface structure; reagents of the second class, which give pronounced departures from the typical order of eliminations, act by attacking particular groups at the centres. It appears to us likely that the average reagent has properties belonging to both classes.

Each centre acts as if it were a separate entity, possessing individual characteristics and, in a word, acting as if it were a separate enzyme. It differs from our usual conception of an enzyme in that it has a limited range of specificity and is inseparable from a surface structure. On such a view it is clearly as impossible for us to state the constitution of a biological active centre as it is to give the constitution of an active centre of a catalytically active alloy from only a knowledge of the constitution of this alloy. Yet it is to be remembered that a relatively small structure or colloidal aggregate may possess an active centre and it would be legitimate to state that the constitution of the structure or aggregate as a whole would be the constitution of the enzyme.

Let us now consider a hypothesis which seems to us to be the most reasonable alternative to that of active centres. The activity of a biological surface may be considered to reside in the specific actions of particular molecules which depend for their stability on a colloidal carrier or on adsorption at a surface. Thus, as soon as surface structure is affected, the molecules become affected, lose their stability and the enzymes are destroyed. Or the effect of a particular treatment may be upon certain of the specifically active molecules adsorbed on the surface. The two views—that of adsorption of specifically active molecules at surfaces which are necessary for their stability and that of active centres—are extremely closely related. It is possible, however, to obtain data which will help us to decide between these views.

IV. SOME CONSEQUENCES OF A THEORY OF ADSORBED SPECIFIC ENZYMES.

On this view we have to imagine that there exist specifically active molecules whose stability or activity is greatly dependent upon a surface to which they are attached. That stability of adsorbed materials can be closely connected with the surface structure is sufficiently obvious from the work of Willstätter and his colleagues. The fact that the stability of an enzyme is so highly dependent on adsorption at a particular type of surface indicates that some particular association of groupings, which can easily be disturbed with resulting inactivity, is responsible for activation. The point we wish to emphasise is that activating power is unlikely to reside solely in one particular group, say $-NH_2$, which may only be primarily responsible for attaching the substrate to the molecule. Now if the specific activating power of the enzyme molecule is dependent on the configuration of this association of groupings we must conclude that any change in the molecule which disturbs this configuration will eliminate its activity. For action to take place, the substrate must be adsorbed at the particular configuration in the molecule, and the amount of adsorption of a substrate at this configuration must be dependent on the nature of the latter. Hence, if the enzymic activity of the configuration remains intact, the power of adsorbing a substrate by the configuration must also remain intact. Any change which alters the adsorption of the substrate must necessarily change the nature of the configuration and hence the latter's enzymic activity. It should be remembered in this connection that the adsorption of a substrate at any part of the surface which is not occupied by the specifically active molecule, or even at a part of the enzyme molecule remote from the active configuration, can have little to do with the activity of the latter. The link must be between substrate and the particular configuration in the enzyme molecule.

Let us now consider that we have a system of two enzymes, **A** which is specifically related to substrate *A*, and **B** which is specifically related to substrate *B*, both *A* and *B* being hydrogen donators. To this mixture of **A** and **B** we add concentrations of *A* and *B* which are at the saturation points of their respective enzymes, *i.e.* at such concentrations that increase in concentration of either *A* or *B* makes no perceptible increase in the velocity of transformation of either of them when taken individually. Then the velocity of reduction due to a mixture of *A* and *B* will be the sum of the velocities due to *A* and *B* taken separately, unless there is preferential or reciprocal adsorption of the substrates on the enzymes. We assume that there are no complications[1] such as side reactions, etc.

Let V_A = velocity of reduction due to A
V_B = velocity of reduction due to B
V_S = velocity of reduction due to $A + B$
$V = V_A + V_B$.

Then, given no adsorption of *B* on **A** or *A* on **B**

$$V = V_S.$$

If $V_S < V$ it is possible to calculate the amount of adsorption of *B* on **A** or *A* on **B**.

1. Let there be only adsorption of *A* on **B**; *i.e.* none of *B* on **A**. Then $V_S = V_A$ + velocity due to unadsorbed portion of **B**.

Therefore velocity due to unadsorbed portion of **B** $= V_S - V_A$.

But velocity due to total **B** $= V_B$.

Therefore fraction of enzyme **B** which is unadsorbed

$$= \frac{V_S - V_A}{V_B},$$

since velocity is proportional to quantity of enzyme.

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[1] In our experiments this is a legitimate assumption, for the amount of methylene blue used is extremely small compared with the amount of donator.

Therefore, the fraction of enzyme **B** which has been inactivated by adsorption of A

$$=\frac{V_B-(V_S-V_A)}{V_B}=\frac{V-V_S}{V_B}.$$

2. Similarly, if there be only adsorption of B on **A**, the fraction of enzyme **A** which has been inactivated by adsorption of B

$$=\frac{V-V_S}{V_A}.$$

3. If adsorption both of A on **B** and of B on **A** occurs, then

(*a*) fraction of enzyme **A** inactivated must be $< \frac{V-V_S}{V_A}$,

and (*b*) fraction of enzyme **B** inactivated must be $< \frac{V-V_S}{V_B}$.

Now if by experiment we can show that there is *no* adsorption of B on **A**, then the amount of adsorption of A on **B** must be given by the expression $\frac{V-V_S}{V_B}$. We can test this by further experiment.

According to the theory of adsorbed specifically active molecules the expression $\frac{V-V_S}{V_B}$ must remain a constant, characterising a property of the enzyme **B** (*i.e.* its power of adsorption of substrate A) and should remain unaltered whilst **B** retains activity. Thus if a surface possessing a quantity of **B** be attacked, so that part of **B** is destroyed, the remainder of **B** should still give the expression $\frac{V-V_S}{V_B}$ as representing the adsorption upon it of substrate A. Or if a portion of the enzyme molecule itself can be attacked without altering the active configuration in it the value $\frac{V-V_S}{V_B}$ should still remain constant. These conclusions depend upon the underlying assumption that a change in the structure of the active configuration of the molecule must inevitably eliminate its highly specific relationship with its substrate. To prevent any misconception of this point, which is important for our argument, it would be well to indicate that an analogy between the enzyme molecule and, let us say, a toxin is fruitless in this connection. It may be urged, for instance, that the action of a toxin may differ in a quantitative way after chemical treatment from that which occurred before such treatment and that this is an argument for supposing that we may also change the activity of an enzyme molecule purely quantitatively by treatment. But the two molecules, toxin and enzyme, must differ in one very important aspect. Both possess, it may be agreed, certain particular groups or associations of groups whose presence is necessary for the demonstration of activity, and both molecules, we may agree for the purposes of argument, are capable of being changed chemically without necessarily altering the configuration of the active groups. But in a toxin molecule, much must depend on the chemical nature of the molecule apart from the presence of the active group, in determining its access to particular structures in the body where it makes its presence demonstrable, *e.g.* permeability of a membrane for the toxin, or the solubility of the latter. Both factors depend on the chemical nature of the molecule as a whole and clearly such factors must influence the quantitative action of the toxin.

Again, the activity of a molecule such as adrenaline is in all probability centred in one or two relatively simple groups or atoms of the molecule, and it is the chemical structure of the molecule as a whole which determines the accessibility of these groups to the susceptible structure of the body.

With an enzyme molecule, however, if a change in the chemical structure does not affect the structure of the active configuration of groups (and this would be surprising in view of the fact that the molecule is very unstable when not attached to an appropriate surface) we would have to consider that the molecule is of such a nature that a disturbance or alteration at one portion of it does not at all affect the active configuration. If this is so it is extremely difficult to perceive how such an alteration could affect the access of a substrate to the configuration itself; it could certainly be argued that if the change were such as to affect the access of substrate to the configuration it must certainly have altered matters to such an extent that the perfect "fit" between substrate and active groups has been rendered less perfect, and hence have produced a cessation of activity. Such a view is fundamental to our understanding of the cause of the high specificity of action obtained between substrate and an enzyme molecule.

We may conclude, then, that, if we can demonstrate the constancy of the expression $\frac{V - V_S}{V_B}$ however the organism or enzyme systems be treated, this will be evidence in favour of the view of adsorbed specifically active molecules. If the expression $\frac{V - V_S}{V_B}$ varies considerably this will be evidence against the view and a more adequate explanation of enzymic activity must be sought.

Experimental results.

The velocities of reduction (reciprocal of reduction times) of mixtures of various substrates are noted in Table VIII, the velocities due to substrates when alone (V_A or V_B) also being given[1]. Care was always taken that the concentration of any substrate was such that increase in concentration made no perceptible increase in velocity of reduction due to the substrate. The values $\frac{V - V_S}{V_A}$ and $\frac{V - V_S}{V_B}$ are worked out and, as explained earlier, represent the relative adsorptions of *B* on **A** or *A* on **B** respectively, assuming that reciprocal adsorptions do not occur[2].

Table IX gives the results obtained with *B. coli* which has been exposed to various treatments so that the mechanism for one or more substrates has

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[1] Since it has been shown [1] that the rate of reduction of methylene blue is linear it is legitimate to take the velocity of reduction as the reciprocal of the reduction time. In our experiments a standard end-point (95 % reduction) is taken, to prevent error due to departure from linearity during the last 5 % of reduction.

[2] It is to be remembered in considering our experiments with *B. coli* that the main site of reduction is at the cell surface.

Table VIII.

Rates of reduction due to donators and mixtures of donators at their saturation concentrations. t_A = time of reduction in minutes due to A. t_S = time of reduction due to mixture of both A and B. Conditions as in Table I.

Concentrations of all donators = $M/28$, except glutamic acid $M/14$ and acetic acid $M/7$.

Exp.	A	B	$V_A = \frac{100}{t_A}$	$V_B = \frac{100}{t_B}$	$V_S = \frac{100}{t_S}$	$V = V_A + V_B$	$\frac{V - V_S}{V_A} \cdot 100$	$\frac{V - V_S}{V_B} \cdot 100$
1	Lactic acid	Succinic acid	18·2	4·3	19·6	22·5	16	67
			13·3	2·1	13·9	15·4	12	71
2	,, ,,	Formic acid	8·3	7·3	13	15·6	31	35
			6·8	9·1	13·7	15·9	32	24
3	,, ,,	α-Hydroxybutyric acid	13·9	8·5	12·5	22·4	71	>100
			7·6	4·6	7·7	12·2	59	98
4	,, ,,	Glucose	9·1	6·2	9	15·3	69	>100
			9·3	5	9·6	14·3	50	94
5	Succinic acid	α-Hydroxybutyric acid	3·3	11·2	11	14·5	>100	31
			4·3	16·7	16·7	21	100	26
6	,, ,,	Formic acid	2·1	14·3	13·5	16·4	>100	20
			2	13·9	13	15·9	>100	21
7	,, ,,	Glutamic acid	5·6	6·6	8	12·2	75	64
			3·3	3·1	3·9	6·4	75	80
8	,, ,,	Glucose	2·1	6·2	8·5	8·3	0	0
			2	3·3	5	5·3	15	9
9	Formic acid	,,	10·5	6·2	15·4	16·7	12	21
			12·2	5·7	15·9	17·9	16	23
10	,, ,,	α-Hydroxybutyric acid	11·1	5·9	12·3	17	43	80
			10·9	5·3	12·5	16·2	34	70
11	,, ,,	Glutamic acid	14·3	2·6	14·9	16·9	14	77
12	Lactic acid	,, ,,	7·3	2·6	8·5	9·9	19	54
13	α-Hydroxybutyric acid	Glucose	5·6	3	7·8	8·6	14	27
			5·9	3·7	8	9·6	27	43
14	Glutamic acid	,,	2·6	6·9	9·1	9·5	15	6
			2·6	6·2	8·2	8·8	23	9
15	,, ,,	α-Hydroxybutyric acid	3·1	11·2	9	14·3	>100	47
			4·4	16·7	14·3	21·1	>100	40
16	Acetic acid	Formic acid	7·1	10	9·5	17·1	100	76
			8·9	14·3	13·3	23·2	100	62
17	,, ,,	Lactic acid	7·3	6·6	9·8	13·9	56	62
			6	8·3	10·9	14·3	57	41
18	,, ,,	Succinic acid	10·3	2	10·3	12·3	19	100
19	,, ,,	Glucose	10	5	12·2	15	28	56
20	,, ,,	Fructose	10·3	7·6	14·3	17·9	35	47
			9·5	6·3	13·3	15·8	26	40
21	Glucose	,,	5·7	11·1	11·1	16·8	100	51
			3·7	6·4	5·9	10·1	>100	66
22	,,	Mannose	5·7	5	5·2	10·7	97	>100
			8·2	11·5	9·5	19·7	>100	89
23	,,	Galactose	5	6	5·3	11	>100	95
			5·4	5·1	5·1	10·5	100	>100
24	,,	Mannitol	5	3·2	4·8	8·2	68	>100
			8·3	4·8	7·7	13·1	65	>100
25	,,	Sorbitol	5·7	3·4	5·9	9·1	57	93
			5·4	2	5·5	7·4	35	95
26	Fructose	Mannose	11·1	5	9·8	16·1	57	>100
			13·3	11·5	14·3	24·8	79	91
27	,,	Mannitol	9·1	4·8	8·3	13·9	62	>100
			10·3	3·3	9·8	13·6	37	>100
28	Mannitol	Sorbitol	2·5	2	2·6	4·5	76	95
			6·5	5·3	6·1	11·8	87	>100
29	Acetic acid	Glutamic acid	9·5	2·6	11·8	12·1	3	11

Table IX.

Velocities of reduction ($V = 100$/reduction time in minutes) due to donators and mixtures of donators with substrates made inactive (or partially so) by treatment of the organism. The treated organism is always washed after treatment and used under similar conditions to the normal organism. Concentrations as in Table VIII.

Exp.	Preliminary treatment of organism	A	B	V_A	V_B	V_S	$\frac{V-V_S}{V_A}.100$	$\frac{V-V_S}{V_B}.100$
1	With 0·005 % $KMnO_4$ soln.	Lactic acid	Formic acid	10	0	9·5	5	—
	,, ,, ,,	,, ,,	,, ,,	8·3	0	8·2	1	—
2	With 1 % KCN soln.	,, ,,	,, ,,	11·8	0	11·8	0	—
3	Exposure to 57° for 1 hr.	,, ,,	,, ,,	0·7	6·4	7·1	0	0
5	With 0·005 % $KMnO_4$ soln.	Glutamic acid	,, ,,	1·8	6·4	4·8	>100	53
6	With toluene	,, ,,	,, ,,	0	9·9	9·3	—	6
7	With 1 % KCN soln.	,, ,,	,, ,,	4·8	1·1	4·3	33	>100
8	,, ,,	,, ,,	Acetic acid	4·8	2·7	4·7	58	100
9	With toluene	,, ,,	,, ,,	0	8	8·5	—	0
10	With 0·005 % $KMnO_4$ soln.	Succinic acid	Lactic acid	0·5	6·4	6·6	60	5
	,, ,, ,,	,, ,,	,, ,,	1·5	10	9·8	>100	17
11	Exposure to 57° for 1 hr.	,, ,,	,, ,,	3·3	1·7	5·5	0	0
12	With 1 % KCN soln.	Glucose	Formic acid	10	0	10	0	—
13	With toluene	,,	,, ,,	0	9·9	10	—	0
14	With 0·005 % $KMnO_4$ soln.	Lactic acid	Acetic acid	6·4	0	5·1	20	—
15	Exposure to 57° for 1 hr.	,, ,,	,, ,,	0·6	3·3	4	0	0
16	With 0·005 % $KMnO_4$ soln.	,, ,,	Glutamic acid	16·7	1·8	13	33	>100
17	Exposure to 57° for 1 hr.	,, ,,	,, ,,	1·7	0	1·87	0	—
18	With 2 % $NaNO_2$ soln. at p_H 6·0 for 1 hr.	Succinic acid	Formic acid	0	6·4	6·4	—	0
19	With 0·005 % $KMnO_4$ soln.	,, ,,	,, ,,	1·5	0	1·5	0	—
20	With 2 % $NaNO_2$ soln. at p_H 6·0 for 1 hr.	,, ,,	Acetic acid	0	7·9	8	—	0
21	With 1 % KCN soln.	,, ,,	,, ,,	3·6	0	3·5	8	—
22	With 2 % $NaNO_2$ soln. at p_H 6·0 for 1 hr.	,, ,,	Lactic acid	0	5·9	4·6	—	28
23	With 0·005 % $KMnO_4$ soln.	Lactic acid	Glucose	6·4	0	5·1	20	—
24	,, ,, ,,	Glucose	α-Hydroxy-butyric acid	0	2·5	2·1	—	16

been eliminated either partially or completely whilst that for others has been retained. From these results it is possible to determine whether the expression $\frac{V-V_S}{V_A}$ or $\frac{V-V_S}{V_B}$ remains constant or not.

For instance, take the case of lactic acid and formic acid.

From Exp. 2, Table VIII,

$$\frac{V-V_S}{V_A}=31\ \%,$$

i.e. assuming no adsorption of lactate on the formic acid enzyme, adsorption of formate effectively eliminates 31 % of the lactic acid enzyme;

or $$\frac{V-V_S}{V_B}=30\ \%\ \text{(average)},$$

i.e. assuming no adsorption of formate on the lactic acid enzyme, adsorption of lactate effectively eliminates 30 % of the formic acid enzyme.

Now from Exps. 1 and 2, Table IX, we find that when the organism is so treated that formate is no longer activated,

$$\frac{V-V_S}{V_A}=2\ \%\ \text{(average)},$$

i.e. there is very little adsorption of formate on the lactic acid enzyme.

Therefore $\frac{V-V_S}{V_B}$ should be about 30 %.

But from Exp. 3, Table IX, we find that when the organism is so treated the lactic acid enzyme is almost completely eliminated,

$$\frac{V-V_S}{V_B}=0.$$

This result is contradictory to the hypothesis of adsorbed specifically active molecules.

Similarly with the cases of
formic acid and glucose (Exp. 9, Table VIII; Exps. 12, 13, Table IX);
lactic acid and succinic acid (Exp. 1, Table VIII; Exps. 10, 11, Table IX);
lactic acid and acetic acid (Exp. 17, Table VIII; Exps. 14, 15, Table IX);
succinic acid and formic acid (Exp. 6, Table VIII; Exps. 18, 19, Table IX);
succinic acid and acetic acid (Exp. 18, Table VIII; Exps. 20, 21, Table IX).

All these results clearly are in opposition to a view which states that the enzymes involved are of highly specific nature and that the fact that substrates do not act quite independently of one another in presence of these substrates is due to adsorptions of inert substrates at the enzymes[1].

But the results are in harmony with the view that active centres on the surfaces are the responsible agents for activation.

Since an active centre has a limited range of specificity it follows that after treatment of an organism, when an alteration of the centre or a rearrangement of its constituent groups has occurred, two effects will be manifest: (1) the configuration will have changed and this will result in some cases in the entire elimination of accessibility of substrates to the centre; (2) the nature of the activating field will have altered. This means that, after treatment, a centre may still retain activity for a particular substrate but yet the accessibility of other substrates to it may have entirely changed. The expression $\frac{V - V_s}{V_B}$ need *not* be constant, and on our views is unlikely to remain constant.

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[1] It should be mentioned, in parenthesis, that if substrates are not used at their saturation concentrations, they appear to act independently of one another. It is highly important, therefore to perform preliminary experiments to ascertain the saturation concentrations.

It is an interesting fact that the experiments of Table IX indicate that in many instances selective elimination of the activating mechanisms, or selective poisoning, reduces adsorption of the inactivated substrate at a centre, where it was previously adsorbed, to a value which is too small to be measured by our experimental method. This would certainly be expected if particular groups or group configurations were associated with the adsorption or orientation of certain substrates, and that these groups or configurations were selectively attacked by the various treatments the organism undergoes.

Table X.

Reduction times of 1 cc. 1/5000 methylene blue soln. due to donators (*A*) at their saturation concentrations in presence of other substances (*B*).

Conditions as in Table I. Normal *B. coli*.

A	*B*	t_A alone	$t_{\text{mixture of } A \text{ and } B}$
Succinic acid *M*/28	Caprylic acid *M*/14	15	33
,, ,,	Nonylic acid *M*/14	15	∞
,, ,,	Adipic acid *M*/84	21·2	29·4
Formic acid *M*/28	Propionic acid *M*/14	9·3	9·7
,, ,,	Caprylic acid *M*/14	9·3	9·0
,, ,,	Nonylic acid *M*/14	9·3	14
Acetic acid *M*/7	Propionic acid *M*/14	10·0	10·5
,, ,,	Caprylic acid *M*/14	10·0	9·5
,, ,,	Nonylic acid *M*/14	10·0	64
Glucose *M*/28	Caprylic acid *M*/14	6·7	∞

It can be shown that retardations of velocities of reduction are effected by substrates which are *not* perceptibly activated, *e.g.* caprylic and nonylic acids (see Table X) so that it is sure that adsorption can occur at active centres of substances which are not activated by them. Again some inactivated donators are adsorbed at active centres as indicated in Exps. 22-24, Table IX. These facts considered in conjunction with the fact that an approximately similar order of eliminations occurs with many diverse treatments (Table III) point, as we have observed elsewhere, to some underlying factor, concerned with the energetics of the field, or with the stability of the centres at the surface, playing a significant part in the eliminations.

V. General considerations.

Formation of active centres at biological surfaces.

There is, naturally, great vagueness at present as to the mechanism of formation of enzymes and their relationship to the remaining constituents of the cells, but it seems to be fairly widely held that the cell is able to elaborate at least two distinct classes of molecules, the very highly specialised molecules which exhibit enzymic behaviour, and the enzymically inert substances which together make up the protoplasmic and histological structures of the cell. This view, which calls for a sharp line of demarcation between the architectural units of the cell, proteins, nucleotides, etc., and the specific enzymes, which are not only being synthesised themselves in the cell but which are regulating the course of metabolism and the growth of the cell itself, seems to be greatly strengthened by the fact that a large number of enzymes can be secreted by the cell. This gives the impression that enzymes are simply products of the cell in much the same way as, let us say, adrenaline.

When it is considered (*a*) that the number of highly specialised enzyme molecules, which the cell is presumed to contain, must be very large indeed and that they must vary considerably in their type and constitution, (*b*) that the evidence, presented in this paper, concerning the dehydrogenations effected by bacteria is contrary to the supposition that activity is due to the presence of many highly specific enzyme molecules, (*c*) that it is extremely difficult to understand how a cell is able to cope with material to which hitherto it has not been accustomed, if its content of specific enzymes, though large, is yet limited, it will be granted that the view stated above stands in need at any rate of some emendation.

The hypothesis we put forward, that enzymic activity may be regarded as the property of the active centres of cellular and intracellular structures (and this includes the smaller structures capable of extraction from or secretion by the cell) leads to a considerable simplification of the above view. Precisely what enzymic behaviour a particular structure or colloidal aggregate in the cell may possess depends on the nature of the active centres which form a part of the structures or of the colloidal aggregates. Enzymes, on this view, are themselves part of and cannot be dissociated from the architectural units of the cell. This does not imply, of course, that only the histological structures are involved; the smaller colloidal aggregates are just as much part of the architecture of the organism. The conditions existing in the cell are such as to bring about just that arrangement or juxtaposition of molecules which makes for the formation of active centres on the normal material of the cell. Thus we may imagine that the protein, nucleotides, etc., are not only so arranged as to form the various substances of the cell but that the arrangement is such that active centres are formed on these particular substances. Enzymes, therefore, and cellular structures are inseparably connected. It is necessary to consider, now, how the active centres are formed.

Let us consider the formation of molecular structures at an interface. This may be accomplished in either of two ways.

1. As new molecules are produced they will orientate themselves in a definite way determined by the forces at an interface and will tend to arrange themselves into a crystal lattice pattern. Now if a number of dissimilar molecules are engaged in forming a surface structure we expect that there will be produced points of weakness or of loose fitting. It is at these points that

stray fields occur, the fields being balanced by surface forces and constituting the active centres of the surface structure.

2. We may conceive of the production of a colloidal aggregate which is represented in a highly diagrammatic manner in Fig. 1. Here various molecules, represented by rectangles, are joined to each other by arrows which represent the connecting links between molecule and molecule. The link may be brought about by electron sharing, or the positions of the molecules relative to each other may be determined by orientating forces in the phases surrounding the molecules. The molecules will tend to take up positions so that the potential energy of the system is at a minimum.

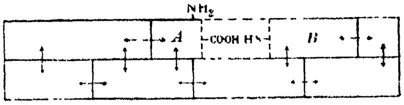


Fig. 1. Illustrating the colloidal aggregate (sectional).

Now let us consider two particular constituents, *A* and *B*, of the aggregate. It may happen that were the molecule *A* free from the restraint of the neighbouring molecules of the aggregate, it would form a structure represented by the skeleton scheme (Fig. 2). Here the external field of the molecule would be at its minimum; "affinities" between atom and atom in it are saturated as far as possible. But when forming part of a structure the action of the other molecules might tend to strain the configuration. This is illustrated in Fig. 3 where we suppose that two constituent groups *M* and *M'* of the molecule *A* are held or attracted by neighbouring molecules in the aggregate, so that a strain occurs, pulling two other constituents *X* and *Y* considerably apart. Such a strain must cause the production of an external field between *X* and *Y*, much greater than that which normally occurs. To take a concrete example,

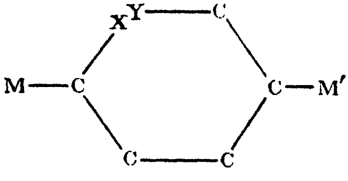


Fig. 2. Illustrating the normal character of molecule *A* with external field between *X* and *Y* at a minimum.

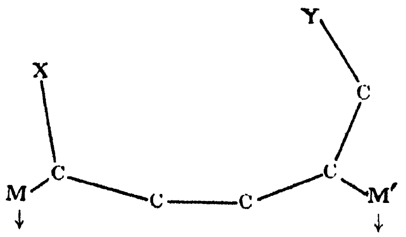
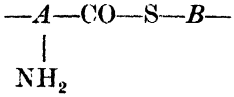


Fig. 3. Illustrating shift in relative positions of *X* and *Y* (with resultant production of increased external field) due to action of surface forces at *M* and *M'*.

in Fig. 1, *X* and *Y* are represented by the amino- and carboxyl groups. These would normally have considerable affinity for each other and tend to approach to a critical distance. The strain imposed by the neighbouring molecules of the surface structure and surrounding phase causes a widening of the distance between the two groups and it is here that we may expect the local production of an external electric field. The carboxyl group at *A* may link with, say, an —SH group at *B*, to form continuity of the surface, but this link, being perhaps much weaker than that normally between carboxyl and amino-group, will not necessarily reduce the field at *A* to any considerable extent.

The active centre we would imagine in the molecule  $-A-\underset{\substack{|\\ NH_2}}{C}O-S-B-$ to be made up at least of the groups $—NH_2$, —CO— and —S— and each of these groups will play its part in rendering a substrate accessible to the centre. The field due to these groupings may not be homogeneous and hence the orientation of a substrate at the centres, so that activation may occur, becomes a highly important consideration.

We have given this illustration of a possible formation of an active centre in order to make clear the difference between the centre and the usual conception of an enzyme. The actual composition of an active centre will in all probability be much more complex than in the illustration given. We may regard the entire aggregate as the enzyme, or the particular centre (at the link) as the enzyme. Each view is equally legitimate. But the residue of the aggregate, distinct from the region occupied by the centre, may be the seat of other active centres, so that the aggregate as a whole may have a much wider range of specificity than were the residue inert in this respect. Such an aggregate would be a relatively large colloidal particle and it would certainly be difficult to regard it as a *specific* enzyme. On the other hand, the residue may be of comparatively small dimensions and contain no other active centres. The specificity of action would be determined by the single centre and the range of specificity may be so small as to make the particle a highly specific enzyme. In this manner we regard the "soluble" enzymes[1] as related to the "insoluble." The distinction between them is simply one of degree.

Stability of active centres.

In the first place there can be little doubt that the centre, represented, let us say, by the illustration (Fig. 1), will have a limited range of stability. Its stability simply rests upon the operation of forces at the interface and we expect any factor which disturbs these forces to result in a disturbance of the links between *A* and *B* and other molecules of the aggregate with the consequent elimination of the centre. Any agent which has the effect of altering surface structure will have a "poisoning" action on the centres, though the poisoning may be selective according to the nature of the centres. We expect the most unstable centres to disappear first, the more stable next and so on. The second type of poisoning action will be on the groupings constituting the centre itself, *i.e.* one of these, say —CHO, may link up with the poison eliminating the access of some particular substrate to the centre. But the field, though changed, may still be capable of activating other substrates accessible to the centre. We expect that such a poisoning action may be reversible.

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[1] It is to be remembered that only heterogeneous catalytic systems are being discussed.

The actual size of the aggregate can give us little information on the stability of the centres. It may be small enough to be classed as a "soluble" enzyme and yet have relatively considerable stability; it may be large enough to be classed as "insoluble" and yet be extremely unstable. We can be sure, however, that any means which will strengthen the forces at the interface in preventing fission of A—B will increase the stability of the centre. There appear to be at least the following ways of accomplishing this.

(*a*) By adsorption of the aggregate at an *appropriate* surface, which may tend to keep the molecules in position and prevent rupture by collision.

(*b*) By the adsorption or combination at the centre, itself, of some substrate, which is reversible in action and which may protect the centre. For instance, adsorption of sucrose by invertase has a protective action on the enzyme.

(*c*) By reducing the chance of rupture of A—B by impact, *e.g.* by increasing the viscosity of the medium in which the aggregate is present.

Co-enzymes.

It is clear that any substance may be classed as a co-enzyme which (*a*) will tend to make a substrate accessible to the centre by combination with, or by facilitating a combination with, the substrate, the resulting combination possessing a lower energy of activation or increased adsorption or a more appropriate orientation at the centre, or which, (*b*) by some action at the centre itself, increases the activating power of the latter or the accessibility of the substrate. Such co-enzymes may not only make activity demonstrable where otherwise it could not be perceived but may increase the range of specificity of the active centre or enzyme.

Anti-enzymes.

We cannot expect that a molecule of the type A—B (Fig. 1), whose enzymic activity is dependent upon surface forces and the nature of the union between A and B, *must* give rise to an antibody which will necessarily eliminate the activity of the enzyme. We may expect the production of antibodies which may act as precipitins, neutralising the charge of the aggregate as a whole, but such combination does not in the least indicate that the active centre will be destroyed.

SUMMARY.

1. An attempt based upon the conception of active centres is made to interpret the mechanism of enzyme action. This conception, which is a development of views put forward earlier [Quastel, 1926] on the mechanism of biological dehydrogenations, is discussed in relation to the specificity of enzyme action and to the behaviour of enzymes when exposed to various changes in chemical and physical environment.

2. Results are given which show that a hypothesis which claims that the activity of surfaces is due to the adsorption thereon of specifically active molecules is inadequate.

3. A suggestion is made as to the mode of formation of active centres (or enzymes) in the cell. It is shown upon what factors their stability depends and in what ways their activity is influenced.

We are greatly indebted to Sir F. G. Hopkins and to Sir W. B. Hardy for their interest in this work and for their ready advice. One of us (W. R. W.) is indebted to the Medical Research Council for a part-time grant.

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CLXIV. THE PHOTOCAPILLARY REACTION OF PLANT SAP.

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Experiments have been carried out on the phenomenon which can be observed when the sap expressed from etiolated seedlings is suddenly illuminated. The surface tension of the sap increases until an equilibrium is attained. This depends on various factors and is destroyed in the dark so that the surface tension decreases.

It cannot be *a priori* stated whether this phenomenon belongs to the photochemical reactions as commonly understood; on the other hand, it is not entirely excluded that the changes of surface tension are brought about by physical forces, so that it is advisable to call the phenomenon a *photocapillary reaction of plant sap.*

The photocapillary reaction was first preliminarily described by the author in a paper on the influence of light on plants in relation to the surface tension of their sap [Herčík, 1926]. It was there stated that etiolated plants give a sap which has low surface tension and which is therefore rich in capillary-active substances. After illumination the amount of capillary-active substances in a plant is more and more reduced so that the plant gives a sap with a higher surface tension. Light therefore increases the surface tension in the plant so that the plant carries out a photocapillary reaction which can be observed more easily *in vitro* if only the expressed sap of etiolated plants is used instead of the plant itself. The sap is found to have a reactivity of the same order as that of the intact plant itself.

METHODS.

Photocapillary reaction was observed in the sap expressed from young etiolated seedlings of *Pisum*, *Vicia*, *Pharbitis* and *Sinapis*. The reaction of *Sinapis* sap being the most intense, young *Sinapis* seedlings were chosen for experimentation.

Seedlings of *Sinapis alba* were cultivated on thick layers of filter-paper in the dark at 20°. Before the experiment they were cut into small pieces and ground in a porcelain mortar with fine sand to a dense pulp. This pulp was pressed out and the sap collected in small watch-glasses with a diameter of 5 cm. The surface tension was measured with the Du Noüy tensiometer, 2 cc. of the sap being used for each measurement. The sap was then illuminated with a strong Osram-Nitra lamp, 500 w., 110 v., from different distances.

The static surface tension of the sap was very soon attained, the sap therefore was illuminated directly after squeezing. The diluted sap also very soon attains its static surface tension which, however, can be very easily disturbed. Therefore several watch-glasses (ten or more according to the number of measurements) were exposed to the light, each glass being used for only one single measurement, so that the surface of the sap was not disturbed by repeated measurements.

EXPERIMENTS.

The experiments were made with sap alone and with sap which had been diluted to various extents with distilled water. The intensity of illumination amounted to 1356 lux.

Table I gives the photocapillary reaction of undiluted sap after illumination with 1356 lux. t' represents time in minutes after exposure to the light and σ surface tension in dynes per cm.

Table I.

Exp. no.	Age of the plants (days)		Changes of surface tension after illumination with 1356 lux										
79	7	t'	0	3	5	7	9	12	17	22	32	52	62
		σ	40·8	43·2	44·4	45·3	46·0	47·1	48·5	47·4	48·0	47·4	47·1
99 *a*	9	t'	0	12	19	27	46	60					
		σ	46·4	46·1	46 8	46·5	46·2	46·1					
93	12	t'	0	5	10	20	25	30	45	60	85		
		σ	38·4	44·3	45·4	45·8	46·3	46·4	47·0	46·4	46·4		
101	16	t'	0	5	10	16	22	34	45	60	121		
		σ	37·2	37·2	39·0	40·3	41·5	42·5	44·1	44·1	44·8		

On Fig. 1 is plotted the reaction curve of Exp. 79. It can be seen that equilibrium is reached after a number of small oscillations. The whole rise of surface tension which the light induces amounts here to 7·7 dynes per cm., in Exp. 99 *a* 0·4 dynes per cm., in Exp. 93 8·3 dynes per cm., and in Exp. 101 7·6 dynes per cm.; the effect which light brings about is therefore comparatively large. The deviation in the case of Exp. 99 *a* is due to the fact that here the initial surface tension of the sap differs greatly from that in the other experiments. It follows that a certain minimum surface tension is necessary for the reaction to begin at all. From other experiments which are referred to in another place [Herčík, 1927, 2] it can be deduced that this minimal surface tension corresponds to about 44·2 dynes per cm.

Control experiments were also made to find out if the sap does not change its surface tension spontaneously in the dark. Table II, in which are recorded some of these experiments, shows that in the dark no change which could be taken into consideration occurs.

Table II.

Age of the plants (days)		Changes of surface tension in the dark			
5	t'	0	20	30	50
	σ	47·0	47·7	47·7	48·3
10	t'	0	42	102	132
	σ	40·5	40 8	41·5	41·9

The photocapillary reaction of diluted sap is different from that of undiluted sap. The surface tension decreases instead of increasing (Table III).

Table III.

Exp. no.	Age of the plants (days)	Concentration of the sap		Changes of surface tension after illumination with 1356 lux					
99 *b*	9	80%	t'	0	11	17	26	45	59
			σ	46·2	45·4	48·9	47 2	45·3	45·1
99 *c*	9	60%	t'	0	9	10	25	44	58
			σ	46·8	45·6	47·8	45·0	43·4	43·3
99 *e*	9	20%	t'	0	6	14	23	42	56
			σ	51·8	48·6	48 3	48·6	47 5	47·8
99 *f*	8	10%	t'	0	5	13	22	41	55
			σ	51·1	48·1	47·1	46·4	45·6	44·9

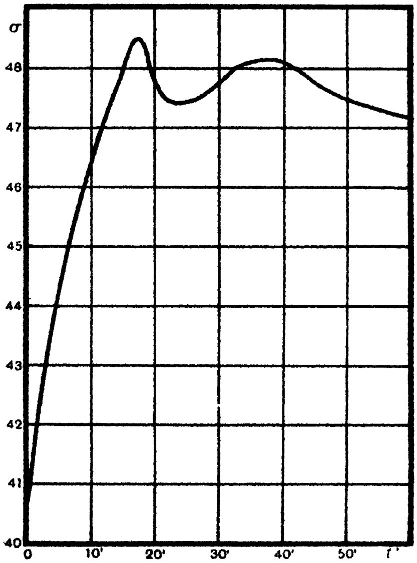

Fig. 1.

From the table it can be seen firstly, that the photocapillary reaction of diluted sap is *in nuce* a reversed photocapillary reaction of concentrated sap, *i.e.* the surface tension decreases; and secondly, that the mode and extent of this decrease depend on the degree of dilution.

In 80 % dilution the surface tension at first decreases a little, then strongly increases and again decreases. In 60 % dilution the first increase is smaller and on the contrary the second decrease is more intense. The 20 % sap shows practically no increase, the surface tension decreasing here after illumination almost regularly. The 10 % sap finally gives a typical negative photocapillary reaction: the surface tension here decreases in a smooth parabolic curve (Fig. 2).

The reaction of slightly diluted sap is therefore composed of both types of photocapillary reaction, of a positive and negative type, indicated by rise and fall of surface tension. The predominance of one of these two types is given by the degree of the dilution. At higher dilutions the negative reaction takes place, at lower dilutions the positive reaction occurs. For other types of the reactions of the diluted sap see the previously mentioned paper of the author [Herčík, 1927, 2].

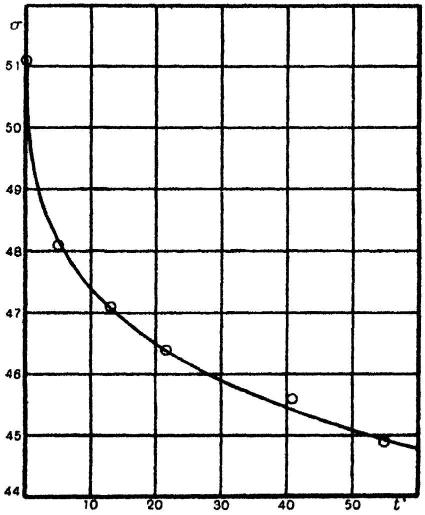

Fig. 2.

In Table IV control experiments are recorded from which we see that the surface tension of diluted sap does not change when the sap remains in the dark.

Table IV. Sinapis alba, 10 *days old etiolated seedlings. Changes of surface tension of sap, diluted to various degrees, in the dark.*

t'	50 %	25 %	12·5 %
0	49·3	51·8	53·7
53	49·7	51·4	53 5

The question naturally arises whether the photocapillary reaction depends in any way on the intensity of the light used. Three different illuminations were taken into consideration, namely of 11,720 lux, 5,420 lux and 13·19 lux. The experiment with the illumination of 11,720 lux is given in Table V.

Table V. Sinapis alba, 12 *days old etiolated seedlings.*

Conc. of the sap	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256
Darkness	44·5	47·4	51·9	51·8	53·8	56·4	63·0	65·3	70·2
Light 60'	48·6	45·8	48·7	50·0	52·2	54·3	58·1	58·3	60·2
Darkness 7½ hours	45·8	45·8	46·3	48·6	50·8	56·3	55·5	60·8	65·0

In this experiment the surface tension of the sap in the dark was first measured. Then the sap was illuminated with 11,720 lux for 1 hour. The surface tension of the undiluted sap increased (only a little because the initial surface tension of the sap was high); that of the diluted sap decreased (in the dilution 1/32 there was no change). After this illumination, the samples were kept in the dark for 7½ hours altogether. This caused a decrease of surface tension of undiluted sap and of the diluted sap in the lower dilutions. In the higher dilutions the surface tension increased.

This shows that with negligible deviations the photocapillary reaction has a reversible character.

On the other hand, a quantitative effect of the higher illumination on the amount of the reaction cannot be observed. The same was established when illuminations of 5420 and 13·19 lux were used. The amount of the reaction in both cases was nearly the same. [See for further detail Herčík, 1927, 2.]

DISCUSSION.

It cannot be maintained that this photocapillary reaction is a photochemical reaction as is generally understood. It is quite independent of the intensity of the light used, it has no induction period and only two decisive factors, namely, the initial surface tension of the sap and the degree of dilution.

The fact that the photocapillary reaction cannot be brought about when the initial surface tension of the sap is relatively high, points to the conclusion that the photoactive substances are highly capillary-active, *i.e.* they determine the lowest limit of the surface tension of the sap.

The degree of dilution strikingly changes the character of the reaction, the positive reaction being converted into a negative one bringing about a decrease of surface tension.

It is reasonable to suppose that the photocapillary reaction is governed by the Hallwachs photoelectrical effect. This suggestion is supported by the following facts. The living substance is known to carry a negative charge. The particles of the sap are undoubtedly also negatively charged. As is known every charge diminishes the surface tension, so that the surface tension of the living substance and also of the sap is reduced according to the amount of the charge present on single particles.

Zwaardemaker and Hogewind [1919] state that the Hallwachs phenomenon can be largely applied for colloidal solutions and therefore also for living substance. This means that the living substance and the sap probably lose their negative charge after illumination and the surface tension rises until an equilibrium is attained. The negative photocapillary reaction can also be explained on the basis of the photoelectrical effect, supposing that the particles lose their charge when the sap is diluted. They are therefore made electroneutral and may be again charged by the action of the light [see Righi, 1888]. The surface tension in this case decreases.

The positive photocapillary reaction which is also carried out by green plants appears to be one of the most important factors in the growth-response of the plants to light. The growth-retarding action of the light can be more easily understood in the following way: by means of photocapillary reaction light increases the surface tension on interfacial surfaces of the cell, and this increased surface tension retards the growth. In the dark the process occurs in the reverse way, the surface tension is reduced and growth is thereby favoured [see Kříženecký, 1927; Bauer, 1924; Kopaczewski, 1924; Herčík, 1926, 1927, 1].

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CLXV. STUDIES ON XANTHINE OXIDASE. IX.

THE SPECIFICITY OF THE SYSTEM. II.

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(*Received September 10th, 1927.*)

One of the most remarkable phenomena connected with enzyme action is the very strict specificity of many enzymes for their substrates.

The oxidising enzymes have not yet been thoroughly studied from this point of view, and an enzyme which lends itself especially well to an investigation of this kind is the xanthine oxidase. The enzyme is easily obtained free from other enzymes and the purine group is particularly well adapted to this study because of the very large number of small changes that can be made in it.

Morgan, Stewart and Hopkins [1922] showed that this enzyme could activate both hypoxanthine and xanthine. They also showed that caffeine (1.3.7-trimethylxanthine), theobromine (3.7-dimethylxanthine), uric acid and guanine (2-aminohypoxanthine) were not activated by the enzyme, but they observed that adenine (6-aminopurine) was slowly oxidised under its influence. They ascribed the action on this last substance to a previous deamination of the adenine to hypoxanthine by some adenase that was present and the subsequent oxidation of the hypoxanthine to uric acid. Dixon and Thurlow [1924] thought, however, that it was probable that there was a direct action of the xanthine oxidase on the adenine and brought some evidence in support of this theory. Following up the above results Dixon [1926] investigated the action of the enzyme on 35 substances of physiological importance, but except for the above-mentioned substances and aldehydes the results were entirely negative.

The enzyme is a particularly interesting one because we can study separately the adsorption of the substrate on the enzyme and also the activation of the substrate by the enzyme, since Dixon and Thurlow [1924] have shown that those purines that are adsorbed inhibit the reduction of methylene blue by hypoxanthine or xanthine, owing to the formation of an interfering film on the surface of the enzyme. Activation of the purine can thus be detected by observing whether the methylene blue is reduced, and, on the other hand, adsorption can be studied by observing the effect of the purine on the velocity of the reduction of the dye by the xanthine.

Thus, by a study of a series of substituted purines, we may hope to determine what structure is necessary for (*a*) activation, and (*b*) adsorption of the substrate.

EFFECT OF STRUCTURE ON ACTIVATION.

The following purines were tested:

3-methylxanthine
8-methylxanthine
9-methylxanthine
1.3-dimethylxanthine
3.8-dimethylxanthine
1-methylguanine
7-methylguanine
1.7-dimethylguanine

and also benziminazole.

The whey preparation of Dixon and Kodama [1926] was used as a source of the enzyme and the results were confirmed using fresh milk. The experiments were carried out in Thunberg tubes in the usual way. The tubes were always evacuated, washed out with nitrogen and re-evacuated before being placed in the bath. The method used in the experiments was as follows.

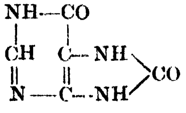
0·5 cc. fresh milk, 5 cc. buffer, 0·5 cc. methylene blue (1 in 1000) and 0·1 cc. of a solution of xanthine containing 5 mg. per cc. were placed in the bath at 40°; decoloration was complete in 120 secs. 2 cc. of the same milk, 3 cc. buffer, 0·5 cc. of the methylene blue and 0·1 cc. of a solution of the purine to be tested containing 5 mg. per cc. were also placed in the bath.

In no case of the above series of purines was there found to be any visible change of colour. Since a change of one quarter of the colour should be readily detected and the observations extended over 4 hours it is easily calculated that, if any of the purines were activated, the velocity of the reaction must have been less than one two-thousandth of that with xanthine.

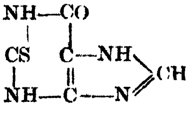
In the above experiment all the solutions of the purines were made up in the following manner. 50 mg. of each was accurately weighed into a 10 cc. graduated vessel. About 2 cc. of water was added and a drop of strong caustic soda. The vessel was agitated and when solution was complete water was added to the mark. Just before use very small quantities of strong hydrochloric acid were added until a cloudiness was apparent and this was just cleared by the addition of minimal amounts of dilute caustic soda. In this way the purines were obtained in a solution which was not alkaline enough markedly to affect the p_H of the reaction mixture.

The significance of the results is discussed later.

Two purines, other than hypoxanthine, xanthine and adenine, have been found which are activated by the enzyme, namely:



6.8-Dihydroxypurine



2-Thioxanthine

0·5 cc. fresh milk, 5 cc. methylene blue (1 in 1000) and 0·1 cc. of the solution of the purine containing 5 mg. per cc. were taken in each experiment.

	Time for complete reduction (secs.)
Hypoxanthine	120
Xanthine	225
6.8-Dihydroxypurine	250
2-Thioxanthine	350

From a consideration of their structure it might be expected that one molecule of the last two purines would reduce one molecule of methylene blue, since they would, in all likelihood, be oxidised to uric acid and thiouric acid respectively. To test this the following technique was devised and very clean-cut results were obtained. 0·15 cc. quantities of the solution of the purine to be tested (5 mg. per cc.) were accurately measured by means of a Pregl pipette into small test-tubes and these were introduced into large Thunberg tubes, which contained 10 cc. of milk and the various under-mentioned amounts of methylene blue. The Thunberg tubes were then evacuated several times, being washed out with nitrogen each time, and when this was complete the tubes were tilted so that the purine was mixed with the milk and the methylene blue, and the tubes were placed in the bath at 40° or 60°. It was found that, when using the 6.8-dihydroxypurine, 4·3 cc. of methylene blue (*M*/1000) were reduced very quickly, 4·5 cc. almost as quickly but that with 4·7 cc. a slight colour persisted for a considerable time. Thus the purine required about 4·6 cc. of the methylene blue and this is in good agreement with the theoretical figure of 4·9 cc. With the thioxanthine, 4·3 cc. was obtained for the titration instead of 4·45 cc. The agreement is excellent and the slight disparity is probably due to unavoidable traces of oxygen.

Xanthine oxidase prepared from ox spleen by the method of Morgan, Stewart and Hopkins was also capable of bringing about the oxidation of these purines. Using about 5 cc. of spleen, 0·5 cc. of methylene blue (1 in 1000) and 0·1 cc. of the purines (5 mg. per cc.) decoloration was complete in the following times.

At 40° (control without purine about an hour):

6.8-Dihydroxypurine	90 secs.
2-Thioxanthine	140

At ordinary temperatures (control without purine more than 8 hours):

Hypoxanthine	4·5 mins.	(4·8)
Xanthine	9	(9)
6.8-Dihydroxypurine	10	(10)
2-Thioxanthine	15	(14)

The numbers in the brackets are calculated from the figures given above when using milk, taking xanthine as the standard of reference. The satisfactory agreement of the velocity ratios shows that the same enzyme is responsible for the activation of all four purines. It is hoped to isolate the products of the oxidation of the 6.8-dihydroxypurine and the 2-thioxanthine.

EFFECT OF STRUCTURE ON ADSORPTION.

In order to determine which of the purines were adsorbed by the enzyme a series of experiments was carried out on each of them. Using a constant amount of xanthine and adding varying quantities of the purine to be tested, inhibition curves were plotted for each purine and these are given graphically (Fig. 1). The exact description of the experiments is as follows. 2 cc. of buffer, 2 cc. of enzyme (2 % whey preparation), x cc. of the purine solution (5 mg. per cc.) and $(2 - x)$ cc. of water were placed in a series of Thunberg tubes. When the series was ready 0·1 cc. of xanthine solution (also 5 mg. per cc.) was quickly added to each, the tubes evacuated as quickly as possible, re-evacuated and placed in the bath at 40°. The time for the complete decoloration of each tube was noted and this was converted into speed by multiplying its reciprocal by 10,000, according to the procedure of Dixon and Thurlow, who also give other inhibition curves.

Discussion.

The results obtained in the experiments on the effect of structure on activation show in a striking manner the high degree of specificity which obtains in this system. The introduction of a single methyl group in either ring is sufficient entirely to prevent any activation, although the inhibition curves show that the enzyme still adsorbs the compounds, but not so strongly as the unsubstituted xanthine. Dixon [1926] showed that the introduction of an amino-group in the 2 position was also sufficient completely to prevent any action, and the introduction of an amino-group in the 6 position (adenine) reduced the velocity to one-fiftieth of that of xanthine.

The fact that 6.8-dihydroxypurine and xanthine are both converted to uric acid at approximately the same rate shows that the two stages of the oxidation take place independently of one another. Particularly interesting is the case of 2-thioxanthine, in which the place of one of the atoms of oxygen in xanthine is taken by an atom of sulphur. As far as the enzyme is concerned, it seems that the sulphur behaves practically in the same way as the oxygen. There is, however, a slight difference in the speed of oxidation—the sulphur compound being oxidised a little more slowly, but this may be due to the molecule not being adsorbed to the same extent.

In the inhibition experiments it seems that the purines can be divided into three classes:

(*a*) those strongly adsorbed:

Hypoxanthine
Xanthine
3-Methylxanthine
Uric acid
Adenine
6.8-Dihydroxypurine
Guanine
1-Methylguanine
7-Methylguanine
1.7-Dimethylguanine

(*b*) those adsorbed to only a small extent:

8-Methylxanthine
9-Methylxanthine
Alloxan

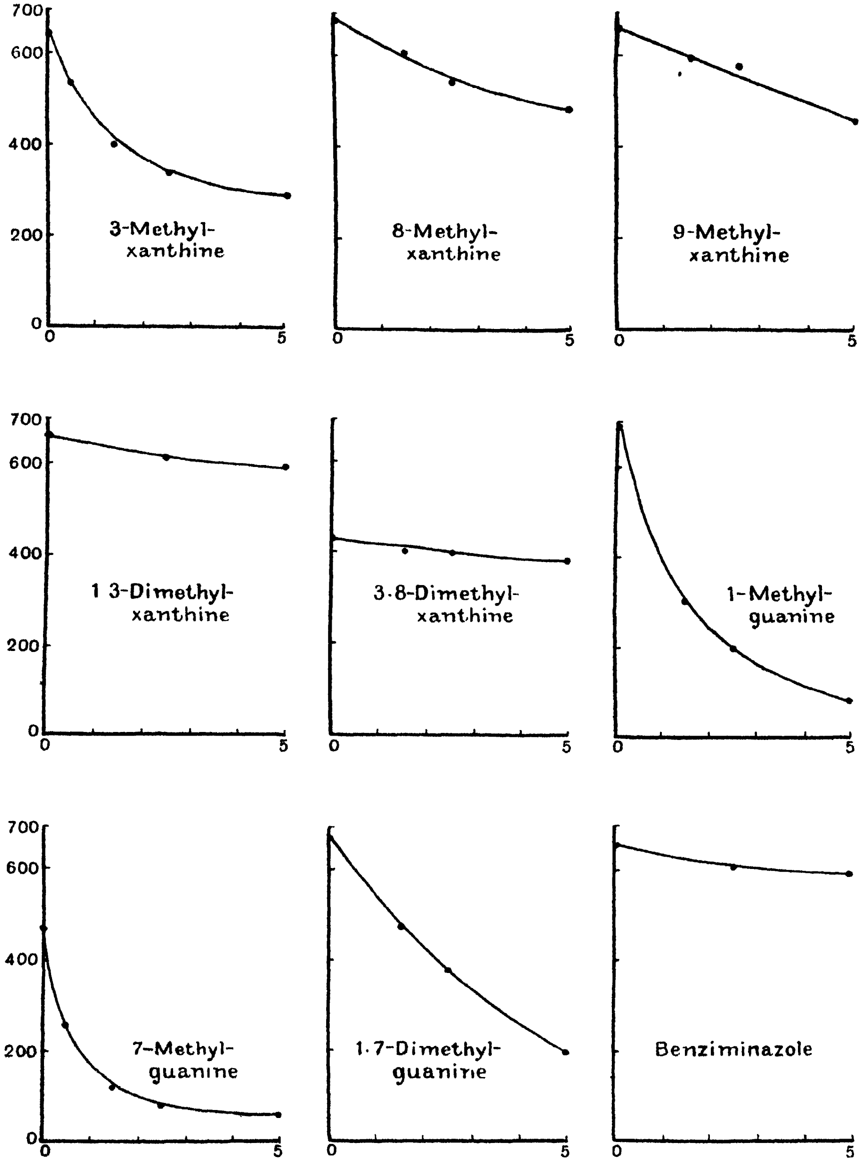


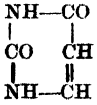
Fig. 1.

Ordinates represent reaction velocity. Abscissae give mg. of purine added (*c*) those not absorbed, or only to a very slight extent:

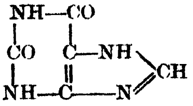
Benziminazole
1.3-Dimethylxanthine
3.8-Dimethylxanthine
Caffeine (1.3.7-trimethylxanthine)
Theobromine (3.7-dimethylxanthine)
Thymine
Cytosine
Uracil
Histidine

(This table also includes the results of other workers.)

It is clear that neither the pyrimidine nor the iminazole ring can by itself cause adsorption of the enzyme—it seems necessary to have the complete purine structure. The pyrimidines are not adsorbed, neither is an iminazole compound such as histidine, and the same is true of benziminazole which contains a benzene ring instead of the pyrimidine ring which is present in the purines. On the other hand, the iminazole ring seems to be very necessary for adsorption and this is very clearly demonstrated by a comparison of uracil and xanthine, because, whereas the latter is strongly adsorbed the former is not.



Uracil



Xanthine

The introduction of a methyl group, particularly in the iminazole ring, tends to prevent adsorption. Thus in the methylxanthines we have much less adsorption than with xanthine itself. The effect of methyl groups in preventing adsorption is very plainly shown when more than one is introduced—these compounds are only adsorbed to a very small extent.

The introduction of an amino-group strongly favours adsorption, so much so as to overpower the effect of the methyl groups. In the guanines the mono-methyl compounds are adsorbed much less than guanine itself but even when two methyl groups are present adsorption is very marked, whereas in the case of the xanthines two methyl groups almost completely prevent adsorption.

It seems likely from the above results that the attachment of the substrate to the enzyme is due partly to the iminazole ring and also partly to the structure of the 1.6-position. The data at present available, interesting and suggestive as they are, are insufficient to afford a basis for a complete theory of the mechanism of adsorption, but it is hoped to complete the above series of experiments so that a satisfactory theory can be evolved.

SUMMARY.

1. The action of the xanthine oxidase on a series of substituted purines has been studied with a view to determining the effect of structure of the purine on (*a*) its activation, (*b*) its adsorption by the oxidase.

2. The enzyme was able to activate only two of these purines, namely, 6.8-dihydroxypurine and 2-thioxanthine.

3. These two purines take up one atom of oxygen for each molecule during their oxidation by this system.

4. The enzyme is very specific, the introduction of a methyl group into the pyrimidine or iminazole ring being sufficient entirely to prevent activation.

5. The complete purine skeleton—the two-ring structure—is necessary for adsorption. The introduction of an amino-group strongly favours adsorption, and the introduction of methyl groups, particularly in the iminazole ring, tends to prevent adsorption.

The author is much indebted to Dr M. Dixon who suggested this line of work to him, and both to him and Mr E. J. Morgan for many suggestions, as well as to Dr Hermann Fischer, Dr H. W. Dudley and Messrs C. F. Böhringer who generously provided him with pure specimens of many of the compounds listed.

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CLXVI. STUDIES ON THE ANTINEURITIC VITAMIN.

I. PRELIMINARY NOTE ON A POSSIBLE SECOND FACTOR.

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(*Received September 13th, 1927.*)

Attention has been drawn by Plimmer and Rosedale [1926] and Plimmer [1926] to the difference in visible symptoms in pigeons on a total deficiency and a partial deficiency of vitamin B in the diet. While the typical paralysis of acute polyneuritis occurred in the former case, shortage in amount of vitamin B led to general debility, the time of appearance depending upon the amount of the vitamin in the ration. The visible effect of shortage is first observed by a falling off of the food consumption, the scattering of the food and the inability of the birds to fly up to the perches of the house. *Post mortem* examinations have shown conditions described by McCarrison [1921], one of the most noticeable being the presence of large masses of decomposing food material in the intestine.

During the course of the preparation of a strong extract of vitamin B from rice polishings a point of interest concerned with the difference between acute polyneuritis and the "shortage" of the vitamin has been observed.

1. Rice polishings have been extracted by 1 % acetic acid, 2 litres being used for each 500 g. of polishings. The extraction is carried on for 8 hours at 40° with constant stirring. After filtering, the extract is concentrated *in vacuo* at 40° to such volume that 1 cc. corresponds to 1 g. of the original polishings. 5 cc. of this extract suffice to cure acute polyneuritis within a few hours. 2 cc. daily, administered to pigeons receiving a diet composed solely of white polished rice, produce steady growth and secure complete maintenance of health and functions.

2. This first extract has been treated with lead acetate until no further precipitate could be obtained, when it has been filtered, the lead being removed by H_2S, which in turn has been expelled by boiling *in vacuo* at 40°. The filtrate and the precipitate after treatment with lead acetate have each separately been subjected to these operations, each being finally made to such volume that 1 cc. corresponds to 1 g. of polishings.

The filtrate portion behaves similarly to the first extract in that 5 cc. will cure acute polyneuritis and 2 cc. given daily will maintain the health of pigeons on a diet of white rice.

2 cc. of the precipitate portion, given daily, enable birds to maintain growth, health and natural functions. This portion, however, will not cure typical acute polyneuritis. The administration of this portion to birds suffering from polyneuritis causes rapid evacuation of the bowel, and, while death ultimately ensues, the usual "stoppage" symptoms are not found on *post mortem* examination. Birds which do not show the typical polyneuritis symptoms, but which have become enfeebled by white rice feeding, are sufficiently relieved by the administration of 5 cc. of this fraction to be able to feed and to remount the perches a few hours after the dose.

In view of this fact, and also on account of the observation that the health of pigeons could be maintained on small quantities, it was thought that small portions of vitamin B had been precipitated by the lead acetate. An amount of this extract corresponding to 2 kg. was concentrated *in vacuo* at 40° and administered in a dose of 20 cc. to a polyneuritic pigeon without cure being effected. This concentration and dosing has been carried out seven times with the same result.

It appears, therefore, that two factors are concerned in the ordinary rôle of vitamin B, one of which has to do with digestive processes, but is independent of the anti-paralysis factor, and is precipitated by lead acetate.

Further chemical and clinical investigations are in progress.

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CLXVII. THE EFFECTS OF COD-LIVER OIL ON THE CALCIUM AND PHOSPHORUS METABOLISM OF THE LACTATING ANIMAL.

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(*Received September 14th, 1927.*)

The demands of the modern dairy animal for calcium and phosphorus are great. The desirability of maintaining a positive balance of these elements has been emphasised recently by Orr [1923], Erf [1923] and Henderson and Magee [1926]. Large amounts of these minerals leave the body in the milk and, where pregnancy and lactation are concurrent, the requirements of the foetus must also be supplied if both the cow and her calf after birth are to be maintained in good condition. The retention of adequate amounts of these minerals is thus a matter of physiological as well as of economic importance. Unfortunately in modern practice the feeding of concentrated foodstuffs, while supplying an abundance of the organic constituents of the diet, will not ensure a sufficient supply of the inorganic elements, since their content of these is, in general, low.

Cod-liver oil is a well-recognised stimulant of calcium and phosphorus assimilation as has been shown by McCollum *et al.* [1922] with rats and by Husband, Godden and Richards [1923] with growing pigs. It therefore seemed of interest to determine whether cod-liver oil would affect the calcium and phosphorus metabolism of the lactating animal in the same way. With that object in view, the following experiments were carried out.

METHODS.

Animals. Goats were used. The procedure was similar to that described by Henderson and Magee [1926].

Feeding. The food consisted of two portions, one of grain and one of hay, the former being made up from a mixture of the following amounts of cereals: 72 lb. ground maize, 28 lb. oatmeal, 14 lb. linseedmeal.

Before the experiment began the animal was fed to satisfaction under the conditions of the experiment and, at the end of a week, a knowledge of its appetite had been obtained. Suitable amounts of grain and hay were then decided upon and the ration was kept at this level throughout the experiment. During the experimental period the oil was mixed with the grain ration and fed in two approximately equal parts in the morning and evening. Distilled water was given *ad lib.* in each experiment.

As far as possible, each experiment was divided into three periods of 16 days' duration, the excreta being collected daily and analysed every 4 days by the following methods:

calcium: precipitation as oxalate and titration with $N/10$ potassium permanganate.

phosphorus: the Pemberton-Neumann method as modified by Richards and Godden [1924].

iodine: a modification by Leitch and Henderson [1926] of Fellenberg's titrimetric method.

milk-fat: ether extraction in a Soxhlet apparatus.

Exp. 1. The experiment began 257 days after parturition and was limited to two periods, a pre-period of 16 days and an experimental period of 20 days, there being no post-period owing to the onset of oestrum.

Diet: 450 g. grain and 900 g. hay per day containing 5·93 g. CaO and 6·39 g. P_2O_5. During the experimental period 10 cc. cod-liver oil were given daily.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1223	1·26	3·65	0·24	5·15	+0·78
Experimental period	1080	1·43	3·20	0·35	4·98	+0·95
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	2·40	3·84	0·05	6·29	+0·10
Experimental period	—	2·38	3·69	0·04	6·11	+0·28

During the oil-period there was an increased retention of calcium in spite of the increased output in both milk and urine. This was accounted for by the decreased output in the faeces, the average daily weight of which was reduced during the oil-period. The balance of phosphorus was also increased, but this was due to diminished excretion in milk and urine, as well as in the faeces.

The average daily milk volume was 550 cc. in the pre-period and 560 cc. in the oil-period. The percentage of calcium (Fig. 1) showed a definite increase immediately after the introduction of the oil, while the phosphorus and fat contents fell as the volume increased.

Exp. 2. The experiment began on the 23rd day of lactation. There were three periods, each of 16 days' duration.

Diet: 300 g. grain and 900 g. hay per day containing 8·49 g. CaO and 6·19 g. P_2O_5. During the experimental period 10 cc. cod-liver oil were given daily.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1066	1·79	7·19	0·05	9·03	−0·54
Experimental period	995	1·46	6·95	0·05	8·46	+0·03
Post-period	1063	1·24	7·14	0·05	8·43	+0·06
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	2·38	4·01	0·02	6·41	−0·22
Experimental period	—	1·80	4·74	0·02	6·56	−0·37
Post-period	—	1·56	4·57	0·02	6·15	+0·04

The balance of calcium showed a marked improvement during the oil period because of reductions in the amounts in milk and faeces. In the post-period the faecal excretion increased, but the amount in the milk continued to fall with the result that the balance became more positive. There was no corresponding improvement in the phosphorus balance in the oil-period for here the faecal excretion reached its maximum. In the post-period the amounts in the milk and faeces fell and the balance became positive. The average daily weight of faeces was reduced during the oil-period and rose again in the post-period.

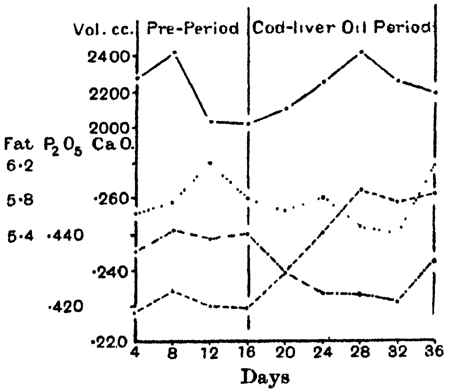

Fig. 1. Volume and percentage composition of milk.

——•—— Vol. .... Fat

–•–•–•–P_2O_5 – – – – – CaO

The milk volume fell throughout the experiment, but the percentages of calcium and fat showed little variation except for a slight fall in the case of calcium in the post-period. The phosphorus content fell definitely from an average of 0·242 % in the pre-period to 0·223 % and 0·222 % in the oil- and post-periods respectively.

Exp. 3. The goat had been lactating for 162 days when the experiment began. There were two periods of 16 days and one of 18 days. The extension of the post-period was necessary because the animal developed diarrhoea and the intake was reduced by making the first two analyses over 5-day periods and giving only 4 days' food. The figures for these 5-day collections were not used in calculating the average excretions and balances.

Diet: 750 g. grain and 1100 g. hay per day containing 9·93 g. CaO and 9·91 g. P_2O_5. During the experimental period 15 cc. of cod-liver oil were given daily.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1782	1·15	8·95	0·92	11·02	−1·09
Experimental period	1405	1·19	7·80	0·71	9·70	+0·23
Post-period	1488	0·92	7·73	0·26	8·91	+1·02
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	2·06	9·12	0·04	11·22	−1·31
Experimental period	—	2·03	7·87	0·04	9·94	−0·03
Post-period	—	1·80	6·56	0·02	8·38	+1·53

In the pre-period the balance of calcium was negative in each analysis, but after the introduction of the oil it became positive with one exception. The diminution in excretion occurred in the faeces, the average daily weight of which again showed a reduction in the oil-period. The balance of phosphorus showed a similar improvement in the oil-period. In the post-period the diarrhoea produced large negative balances of both minerals and the succeeding analyses showed positive balances.

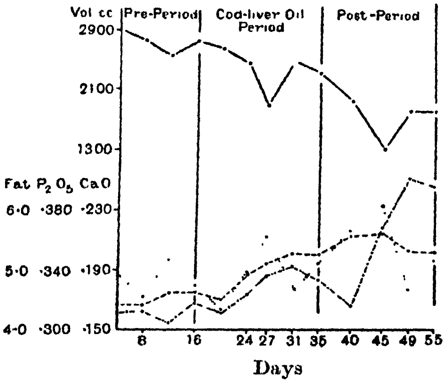

Fig. 2. Volume and percentage composition of milk.

——•—— Vol. .... Fat
–•–•–•– P_2O_5 – – – – – CaO

During the oil-period the milk volume was slightly lower than in the pre-period, and in the post-period it was much more so owing to the diarrhoea (Fig. 2). When this passed off the volume rose, but never regained its former level. In the oil-period the percentage of calcium increased steadily, but reached its maximum in the post-period when the volume was at its minimum.

The phosphorus content also increased in the oil-period but, after a slight fall, rose much higher in the post-period. The percentage of fat showed wide variations and also reached its maximum in the post-period when the volume was lowest.

Exp. 4. The special objective of this experiment was to determine whether the increases in the calcium content of the milk in the oil-period of Exps. 1 and 3 were related to the stage of lactation. The animal was the same as in Exp. 1 and the experiment began on the 5th day of lactation. There were three periods of 16 days. Only the milk was analysed.

Diet: 500 g. grain per day with hay *ad lib.* 10 cc. of cod-liver oil were given daily during the experimental period.

Results: the average daily volume for the three periods was, pre-period 928 cc., oil-period 771 cc. and post-period 454 cc. The calcium content was unaffected by the oil, the average figures for the three periods being 0·220 %, 0·223 % and 0·265 %. The phosphorus percentage also showed little change during the oil-period: pre-period 0·335 %, oil-period 0·337 % and post-period 0·365 %. The average fat content was lowest in the oil-period, falling from 5·98 % in the pre-period to 5·56 % in the oil-period, and rising to 7·22 % in the post-period. It will be noted that all three constituents rose markedly in the post-period as the volume fell. In this experiment the iodine content was also estimated with the following results for each analysis:

Pre-period	13,	13,	13,	11γ %.	Av. 12·5,
Oil-period	17,	17,	17,	19γ %.	Av. 17·5,
Post-period	16,	15,	14,	13γ %.	Av. 14·5,

where $\gamma = 0{\cdot}000001$ g.

The average total amounts of iodine secreted in the milk per day were: pre-period, 116γ; oil-period, 135γ; post-period, 66γ. Thus the feeding of 10 cc. of cod-liver oil which contained 80γ of iodine increased both the percentage and the total iodine contents of the milk.

Exp. 5. Kelly [1925] has shown that with growing pigs the addition of small quantities of iodine as potassium iodide to the diet results in an increased retention of nitrogen and phosphorus, but has less definite effects on that of calcium. This suggested that the beneficial effects of cod-liver oil might be attributable to its high iodine content. An experiment similar to Exp. 3 was accordingly carried out in which the same animal was given an equal volume (15 cc.) of olive oil plus an equivalent amount of iodine (120γ) as potassium iodide. The experiment began on the 229th day of lactation; the pre-period and experimental periods were of 16 days' and the post-period of 12 days' duration.

Diet: 400 g. grain and 1200 g. hay per day containing 20·52 g. CaO and 9·85 g. P_2O_5. During the experimental period 15 cc. of olive oil and 120γ of iodine as potassium iodide were mixed with the grain every day. The large calcium intake was due to an unusual amount of clover in the hay.

Results:

Average daily excretion and balance in g.

	Weight of faeces	Output of CaO				Balance of CaO
		Milk	Faeces	Urine	Total	
Pre-period	1319	0·99	17·77	0·12	18·88	+ 1·64
Experimental period	1290	0·81	19·00	0·09	19·90	+ 0·62
Post-period	1243	0·59	20·35	0·08	21·02	− 0·50
		Output of P_2O_5				Balance of P_2O_5
Pre-period	—	1·81	5·57	0·03	7·41	+ 2·44
Experimental period	—	1·41	8·39	0·03	9·83	+ 0·02
Post-period	—	0·89	9·50	0·03	10·42	− 0·57

The balance of both calcium and phosphorus fell greatly during the experimental period as a result of greatly increased excretion in the faeces. The average daily weight of faeces fell throughout the experiment, but there were increases in the percentages of both calcium and phosphorus. In the post-period assimilation remained poor and the balances were, on the average, negative.

The milk volume fell continuously throughout from a daily average of 437 cc. in the pre-period to 326 cc. in the experimental period and 225 cc. in the post-period. During the experimental period the percentage of calcium rose steadily and remained high in the post-period, the average percentages being 0·227 %, 0·248 % and 0·264 % in the respective periods. The percentage of phosphorus also reached its maximum in the experimental period, the average figures for the period being 0·414 %, 0·434 % and 0·394 %. The fat percentage remained steady in the pre-period and in the first half of the experimental period, but rose to its maximum in the second half of that period and fell again in the post-period. The average iodine contents for the periods were: pre-period, 17 γ %; experimental period, 23 γ %; post-period, 22 γ %. The average daily total iodine secreted in the milk was the same in the pre- and experimental periods, but fell markedly in the post-period.

Exp. 6. Exps. 1–4 indicated that the fat content of milk was not definitely affected by cod-liver oil in the amounts given, and in Exp. 5 the feeding of olive oil and potassium iodide gave an increased fat content in the second half of the experimental period. Further, the feeding of iodine in either organic or inorganic form produced definite increases in the iodine content of the milk. It was therefore decided to determine the effects of olive oil alone on the composition of the milk, in order to find whether the effects observed in Exps. 4 and 5, *viz.* increase in iodine and fat contents, could be attributed to the oil *per se*.

The animal used was the same as in Exps. 1 and 4 and the experiment was of the same length as Exp. 5. It began on the 69th day of lactation and only the milk was analysed.

Diet: 400 g. grain per day with hay *ad lib.* In the experimental period 15 cc. of olive oil were given per day.

Results: the milk volume rose throughout the experiment, the average daily figures being: pre-period, 537 cc.; experimental period, 605 cc.; post-period, 729 cc. The average percentages of calcium and fat were highest in the oil-period, but the average phosphorus percentage fell throughout; the average iodine content and total iodine were both at a minimum in the oil-period. These figures are given in the following table.

	CaO %	P_2O_5 %	Fat %	Iodine γ %
Pre-period	0·245	0·387	6·19	16
Experimental period	0·251	0·337	6·90	13
Post-period	0·234	0·362	6·33	14

DISCUSSION.

Each of the cod-liver oil experiments shows a reduction in the average daily weight of faeces during the oil-period as compared with the pre- and post-periods. That this was not due to progressive constipation as a result of confinement is shown by the rise in the post-period in one case to within a few g. of the pre-period amount. This fall in the weight of faeces was accompanied by an increase in the percentage of calcium immediately after the introduction of the oil. In Exps. 2 and 3 the average percentage of calcium in the faeces was higher in the oil-period than in the pre-period and in Exp. 1 it fell by only a very slight amount.

In each of these experiments, however, there was an improved balance of calcium during the oil-period. In Exps. 1 and 3 this was completely accounted for by the reduction which took place in the faecal excretion; for in these there was an increased excretion in the milk while changes in the urinary excretion were comparatively slight. In Exp. 2 the amount of calcium in the faeces was also reduced, but the improved balance was not entirely due to this, for the amount in the milk was also falling.

This improved balance of calcium as a result of feeding cod-liver oil agrees with the work of Hart, Steenbock and Hoppert [1921], who found that with both lactating and dry goats the amount of calcium in the faeces was reduced.

The phosphorus balances, on the other hand, did not show a similar improvement. In Exps. 1 and 3 the increased retention was almost wholly the result of a reduction in the faecal excretion. The second experiment, however, showed no such improvement, as the faecal excretion was at its maximum in the oil-period; but it may be noted here that this animal was at a different stage of lactation from the others and showed other differing results.

The results with regard to calcium and phosphorus balances are not in agreement with those of Meigs [1926], who found no improvement in the retentions of either of these minerals or of nitrogen after feeding 100 cc. daily to cows.

Numerous experiments have been made in attempts to increase the concentration of a particular constituent of the milk by feeding excessive amounts of it in the diet, but these have generally failed [Lauder and Fagan, 1914;

Denis, Sisson and Aldrich, 1922; Mattick and Wright, 1925]. Exps. 1 and 3, however, show that, where the absorption of calcium was increased by feeding cod-liver oil to animals in the later stages of lactation, the calcium content of the milk was increased. The benefits of increased calcium assimilation would thus be shared by both mother and offspring, since some of the extra calcium absorbed would be available for the young. It is of interest in this connection, as shown by Telfer and Crichton [1924], that even small changes in the mineral composition of goat's milk may affect the nutrition of young kids. In the case of the animals in early lactation (Exps. 2 and 4), there was no such increase in the calcium content of the milk.

Iodine, however, differs from the other mineral constituents of milk in the ease with which its concentration may be increased by feeding. This increase has been shown to occur when iodine is given either in what is presumably organic combination in cod-liver oil, or in inorganic form as potassium iodide. These findings confirm the results of earlier experiments at this Institute [Orr and Leitch, 1927]. Preliminary trials by dialysis and ultra-filtration also showed that, of the total normally occurring in goat's milk, about 80 % was in ionic form and that, when the increase took place after either cod-liver oil or potassium iodide, the relative proportions of ionic and colloidal iodine remained unaffected.

The effects of cod-liver oil on the fat content were much more indefinite. Exp. 1 showed a reduced percentage in the oil-period but with the rise in volume the average daily total secretion of fat remained the same. In the second experiment changes in the fat content were insignificant, while in the third it varied inversely as the volume. In Exp. 4 it was lowest in the oil-period, but in this experiment with the fall in volume in the post-period there was a marked rise in the fat content. These results are not in agreement with those of Drummond *et al.* [1924] and Golding, Soames and Zilva [1926], who concluded that cod-liver oil produced a reduction in the yield of milk-fat which was greater than might be attributed to the natural decline with advance of lactation; they are rather in agreement with those of Taylor and Husband [1922], who stated that, after feeding rations high in each of the constituents, protein, carbohydrate and fat, the percentage composition of milk was determined by its rate of secretion and that diet had no direct influence on it, but affected it only indirectly through changes produced in the daily volume.

The single experiment in which cod-liver oil was replaced by an equal volume of olive oil and an equivalent amount of iodine as potassium iodide did not show that the beneficial effects of cod-liver oil could be reproduced by such a change. Although the addition of small amounts of potassium iodide to a cereal ration fed to growing pigs produced increased balances of nitrogen and phosphorus [Kelly, 1925], it must be emphasised that the amounts of iodine were greatly in excess of those which had previously been given in cod-liver oil by Husband, Godden and Richards [1923]. In this experiment and in the one in which olive oil was given alone there was an increase in the fat content of the milk in each case in the second half of the experimental period. These results are complicated by the fact that in one case the volume was falling and in the other rising and it does not seem possible to ascribe the effect to either the iodine or the oil. The olive oil experiment, however, shows that the increase in the iodine content in Exp. 5 must have been due to the potassium iodide and not to any effect of the oil *per se*.

SUMMARY.

1. The feeding of cod-liver oil to goats improved the balance of calcium by reducing the amount excreted in the faeces. The effects on phosphorus excretion were variable.
2. In the case of animals in late lactation, the percentage and total amounts of calcium in the milk were increased by feeding cod-liver oil.
3. The amount of iodine in the milk was increased by feeding either cod-liver oil or potassium iodide.
4. The fat content of the milk showed no definite changes as a result of feeding cod-liver oil.
5. In a single experiment in which cod-liver oil was replaced by olive oil plus an equivalent amount of iodine as potassium iodide, the effects of the former were not reproduced.

To Dr J. B. Orr, Director of the Rowett Research Institute, and Dr H. E. Magee, Head of the Physiology Department, my thanks are due for their kind help and criticism. I am also indebted to Miss A. E. Glennie for assistance in the analytical work.

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CLXVIII. THE GLUTATHIONE CONTENT OF CHICKEN SARCOMA: A REJOINDER

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(Received September 19th, 1927.)

THE recent paper on "Quantitative studies of the nitroprusside reaction in normal tissues and tumours," by Kennaway and Hieger, appearing in this *Journal* [1927], seems to call for a brief comment.

A year ago we published our observations on the same subject [Yaoi and Nakahara, 1926] and reported that the Rous chicken sarcoma contains but little glutathione. This fact seemed especially interesting because of the abundant occurrence of glutathione in transplantable mammalian tumours, such as rat sarcoma and rat and mouse carcinomas, which are known to be similar to the avian tumour as regards their rate of tissue respiration and glycolysis [Murphy and Hawkins, 1925]. Kennaway and Hieger alleged that our results are incorrect, on the basis of the supposition that we used "glutinous and largely non-cellular material" from the centre of the overgrown tumours, instead of actively growing sarcoma tissue. This allegation is totally unfounded.

Nobody who is at all familiar with experimental cancer research would think of using central necrotic material for any study of tumour tissue. In our experiments we took the actively growing peripheral layer of the tumour, taking care not to include any adherent muscular tissue. The practice is so universally adopted by all experienced workers that we did not feel constrained to mention it in our paper.

In view of the quoted allegation, it is important to compare our figures. We reported three determinations, namely: 12, 13 and 20 mg. glutathione per 100 g. of fresh sarcoma tissue, based on the starch method. Kennaway and Hieger's figures for the same method ran: 9·6, 14, 20, 24, 27 and 54 in the case of intramuscularly implanted tumours, and 18, 21, 21, 24, 29, 30, 41, 47, 51 and 52 in those subcutaneously implanted.

We would call attention to the very great variation of their figures. Even allowing for a considerable experimental error, we can hardly see how such a wide range, as from 9·6 to 54 mg. %, for example, can be obtained by using any uniform material. From the information given it appears that they used only very young tumour grafts (6–11 days old) for their test. It is probable that their higher figures are attributable to the admixture of the reactive

With the addition of 0·2 % of ergosterol, the three bands were detectable with certainty, while lesser amounts gave positive results, although these results were not always unequivocal. The sensitivity of the spectrographic method will depend primarily on the transparency of the oil under investigation, and in many cases of clear samples of high purity, percentages as low as 0·01–0·02 have been detected, *e.g.* cottonseed oil.

In what follows a "negative" test does not, therefore, *exclude* a concentration of ergosterol less than 0·2 %.

The reaction, ergosterol ——(light)——→ vitamin D is accompanied by the appearance of a new band at 247 $\mu\mu$ which is, in all probability, characteristic of the vitamin [Morton, Heilbron and Kamm, 1927]. Unfortunately a very considerable concentration of pure vitamin (of the order of 0·01 g. per 100 cc. alcohol) is necessary to secure the detection of this absorption band. In view of the fact that such a concentration is equivalent to some thousands of doses of cod-liver oil (in respect of vitamin D), it is not surprising that the test will not serve as a criterion of the absence or presence of the antirachitic vitamin. As a further indication of the limits of the spectroscopic method, it may be pointed out that cod-liver oil, the source of our original "active" cholesterol, does not exhibit the pro-vitamin bands, although it does, of course, contain the precursor substance. A pronounced inflection, only, is shown in the same region as the ergosterol bands. It will thus be seen that the method only enables us to discriminate between oils containing relatively little ergosterol, and those containing quite reasonable amounts.

A large number of oils have been subjected to spectrographic examination, and the results are summarised in Table I. (See also Figs. 1—5.)

Certain of these oils will now be discussed in more detail.

Cottonseed oil. The unsaponifiable matter obtained by the saponification of a large quantity of oil, gave a high yield of crystalline sterol, which was fractionally crystallised from ethyl acetate, the various fractions then being examined spectroscopically. The least soluble fraction was found to contain 2·5 %, the most soluble 0·75 % of ergosterol. Cottonseed oil would thus appear to be a very rich source of this compound.

Sesame, illipi nut, shea nut and pumpkinseed oils. These all show well-defined selective absorption in the middle ultra-violet. Since, however, these bands do not exhibit the fine structure characteristic of ergosterol, there is no valid reason for connecting them with the pro-vitamin. Moreover, a preliminary examination of the unsaponifiable matter of shea nut oil has so far given no indication of the presence of ergosterol.

Yeast-fat. As would be expected from the work of Smedley MacLean and Thomas [1920], who found it to contain as much as 40 % of ergosterol, this fat gave by far the most pronounced absorption of all the oils examined.

Ground nut oil. This was probably the most interesting oil examined, showing as it did, big variations from sample to sample. In some cases there was no sign of selective absorption, in others very well-defined ergosterol bands were obtained. Moreover, in all cases where the ergosterol bands were plainly present, there was also a more or less well-defined band in the neighbourhood of $320\mu\mu$. This latter band has only been found hitherto in oils of animal origin such as wool-fat, whale oil, cod-liver oil, etc. [cf. Schlutz and Morse, 1925; Heilbron, Kamm and Morton, 1927], and there is no definite evidence of its connection with vitamin chemistry. Experiments are, however, now in progress, which have been designed to elucidate the nature of the compound exhibiting this band.

Table I.

Oil	Ergosterol bands	Band at or near $320\ \mu\mu$	Remarks
Olive	–	–	Band at λ max. $=407\ \mu\mu$
Nux vomica	–	–	
Tiger nut	–	–	
Alfalfaseed	Very feeble	–	
Coconut	–	–	
Babassu	–	–	
Yeast-fat	+ + + + +	–	
Sesame	?	–	Well defined band at λ max. $=287\ \mu\mu$
Kapokseed	–	–	
Rape	–	–	
Soya bean	–	–	
Mowraseed	Inflection 265-280 $\mu\mu$	–	
Illipi nut	?	–	Selective absorption λ max. $=270\mu\mu$
Shea nut	?	–	„ λ max. $=278\mu\mu$
Cottonseed	+ +	–	
Linseed	+ +	–	
Maize	+ +	–	
Ground nut sample A	Inflection	–	
„ „ B	–	–	
„ „ C	–	–	
„ „ D	+	–	
„ „ E	+ +	+	
„ „ F	+	–	
„ „ G	+ +	+ +	
„ „ H	+ +	+ +	
Brazil nut	–	–	
Palm oil	–	–	
Palm kernel	–	–	
Rice meal	–	–	
Pumpkinseed	?	–	Well defined selective absorption λ max. $=276{\cdot}5\ \mu\mu$. No fine structure
Sunflower	–	–	
Whale	–	?	
Wool-fat	Inflection	+ +	
Cod-liver oil	?	+	
Ditto. Unsap.	?	+	
Extracted C.L.O.	?	+ +	
"Ostelin"	?	+	

Olive oil. Lewkowitsch [1927] examined the absorption of olive and tea oils. A band at $275\mu\mu$ was observed in the former oil. In view of the frequent contamination of olive oil with cottonseed oil, and also since a sample known to be free from adulterants, examined by us, did not show any band, the observation of Lewkowitsch should be accepted with reserve.

The activation of oils by irradiation bears a close relation to the stimulation of certain vital processes in phototherapeutics. It is well known that skin is rich in cholesterol and that there is reason to suppose that a part at least of the mechanism of light therapy is the formation of vitamin D in the epidermis. This has not yet been proved to be true.

Thin sections of skin have been obtained by us (by the kindness of Prof. Blair Bell) and examined spectroscopically. The results were in approximate agreement with the data of Glitscher and Hagenbach [1926]. Sections of 0·1 mm. were not transparent below 297 $\mu\mu$, and with thinner sections it was difficult to retain continuity when the sections were placed between quartz plates. Several experiments with very thin sections were carried out, and although inflections were shown in the middle ultra-violet regions of the curves, more definite evidence of selective absorption could not be obtained. Such a result is not surprising as the concentration of ergosterol in the epidermis must in any case be exceedingly small.

SUMMARY

1. A method of detecting and estimating ergosterol in oils by direct spectrographic examination has been studied and its limits have been defined.
2. A large number of oils and oil extracts have been examined, and their absorption curves have been obtained.

In conclusion we desire to express our thanks to Messrs J. Bibby and Sons, Ltd., to Messrs Joseph Nathan & Co., Ltd. (proprietors of Glaxo), and to Messrs R. Silcock and Sons, Ltd., for samples of oils, and to the Food Investigation Board of the Department of Scientific and Industrial Research for a grant which has enabled the work to be carried out.

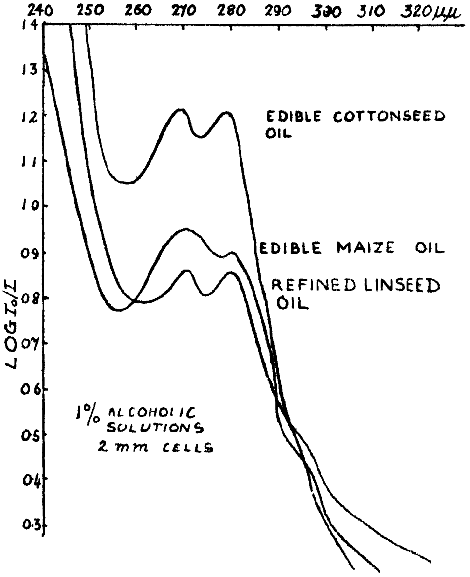

Fig. 1

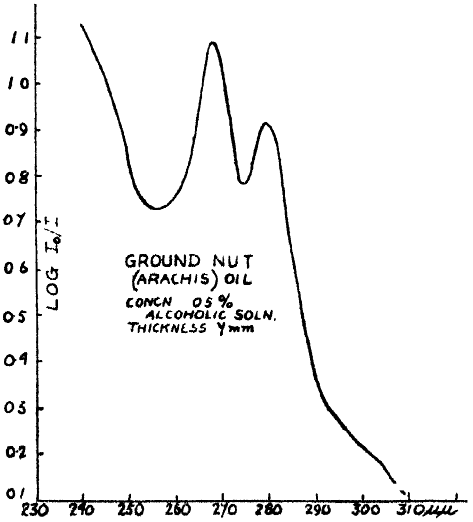

Fig. 2.

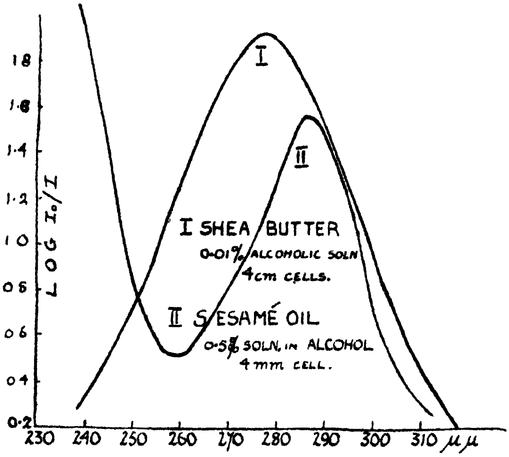

Fig. 3.

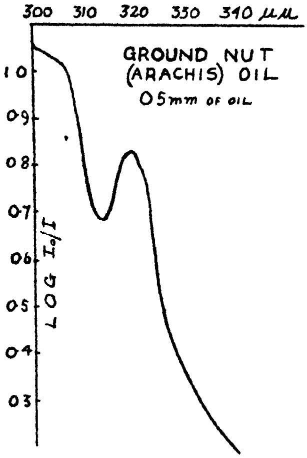

Fig. 4.

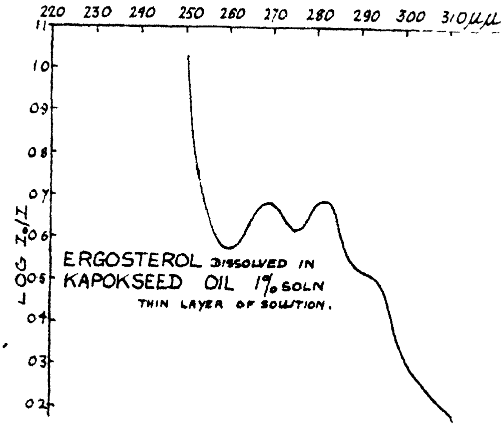

Fig. 5.

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CLXX. INVESTIGATIONS ON GELATIN.

PART IX. THE SCISSION OF GELATIN INTO CONSTITUENT PROTEINS.

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(*Received October 5th, 1927.*)

Recent investigations carried out in this laboratory indicate that gelatin consists of molecules, or aggregates of molecules, which readily undergo intramolecular change. It has been shown, for example, by Knaggs [1923] that if gelatin is hydrolysed rapidly by throwing it into boiling 25 % hydrochloric acid solution, it yields a distinctly smaller fraction of basic products precipitable by phosphotungstic acid than it yields when it has been allowed to stand with the cold acid solution before boiling. This phenomenon has been further investigated by Schryver and Buston [1927], who have found that the increased amount of basic product obtained by treating the gelatin with cold acid before hydrolysis is due mainly to the presence of *dl*-lysine, which cannot be ascribed merely to the racemisation of the *l*-lysine, since the latter occurs along with the optically inactive base in normal (or slightly more than normal) quantities. Thornley [1927] has found that the treatment of gelatin with relatively dilute cold solutions of acids or alkalis can give rise to changes in the amounts of basic products obtained when the sample thus treated is afterwards submitted to rapid hydrolysis with strong acids. Furthermore, Knaggs and Schryver [1924, 1] have shown that when gelatin is heated in water and then hydrolysed by boiling acids in the usual way, there is in the hydrolysis products more nitrogen in the form of non-amino-nitrogen than in those obtained in a similar way from gelatin not previously heated with water—indicating that this comparatively mild treatment causes the formation of stable nitrogenous rings, which are not opened up by subsequent hydrolysis with strong acids.

ELECTROLYSIS OF GELATIN HYDROCHLORIDE AND SODIUM GELATINATE.

In view of the accumulating evidence of the readiness with which structural change takes place within the molecules, it was of interest to ascertain whether gelatin possesses the same structure when functioning as anion and as cation.

Some evidence has been afforded by the investigation of ultra-violet absorption spectra by Baker and Davidson [1925] that two distinct forms of gelatin exist. The experiments recorded in this communication tend to confirm the conclusions drawn by these authors.

For the purposes of the experiments, a gelatin was employed which had been freed from ash and separated from simpler degradation products, which are present in ordinary commercial samples, by flocculation in an electric field [Knaggs, Manning and Schryver, 1923; Knaggs and Schryver, 1924, 2]. The flocculum thus obtained was thoroughly washed on a filter with cold water. Two portions of this were taken and dissolved in water; to one solution hydrochloric acid was added to bring it to p_H 3·0, and to the other sodium hydroxide to bring it to p_H 8·0 or 9·0. The gelatin was precipitated from these solutions by electrolysis, the poles being separated from the gelatin solutions by means of semi-permeable membranes[1].

When the gelatin was deposited cationically by the electrolysis of the hydrochloride, the lower pole of the apparatus was made the cathode; when, on the other hand, it was deposited anionically by electrolysis of sodium gelatinate, the lower pole was the anode. The upper poles were washed with a continuous stream of CO_2-free water. Electrolysis was carried out with very small currents, which hardly ever exceeded 10 milliamps., and were generally appreciably less. Noticeable heating effects were thereby avoided. The electrolysis was continued until the washings of the upper pole showed no traces of chlorine, or of alkali. At this stage, with a potential fall of 20–30 volts per centimetre, the current passing had usually fallen below 1 milliampere.

The difference between the appearance of the gelatin deposited cationically and that deposited anionically was extremely well marked. The former separated as a compact, almost transparent gel on the collodion membrane, from which it could be peeled off as a single solid mass by a knife. The gel occupied only a relatively small bulk of the whole system, and the supernatant liquid was water-white and clear; it contained only a small amount of nitrogen, which amounted to about 3·5 % of the total originally present in the solution. The gelatin deposited anionically formed, on the other hand, a bulky flocculum extending from one-half to two-thirds of the way up the cylinder, while the clear liquid in which this flocculum was suspended was relatively rich in nitrogen, containing nearly 18 % of the total. (See Experimental Details, Series A.)

By the electrolysis of sodium gelatinate, therefore, a considerable portion of the nitrogen remains unprecipitated, and two distinct fractions are thus obtained, *viz.* a soluble and an insoluble.

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[1] The apparatus employed was similar to that figured in the *Biochem. J.* 1924, **18**, 1082. Simple cylinders were used instead of bell-jars, and a number of such units were placed either in series or in parallel, so that several experiments could be carried out at one time. By a suitable arrangement of plugs and switches the current passing through any unit could be measured when required. Platinum electrodes were employed in all cases.

The insoluble ana-fraction.

The insoluble fraction, provisionally designated insoluble ana-gelatin, carries down with it a certain amount of the soluble fraction—soluble ana-gelatin—which at the conclusion of the electrolysis is not distributed evenly throughout the liquid, but tends to pass with the insoluble fraction towards the anode. Most of the soluble portion is, however, readily removed from the insoluble ana-gelatin by washing. If the latter be shaken thoroughly with ice-cold water, and the mixture be then centrifuged, a certain amount of nitrogen will be found in the supernatant liquid. If these processes of washing and centrifuging be repeated several times, it will be found that the nitrogen in the supernatant fluid rapidly diminishes with successive treatments until after three or four such treatments it becomes approximately constant, amounting to about 3 mg. per 100 cc. of the liquid. This must be taken as a measure of the solubility of this fraction in water, and it indicates that this so-called insoluble fraction is soluble in water to the extent of about 15 parts in 100,000. (See Experimental Details, Series B.)

The soluble portion removed by these washings may not represent the total amount of gelatin in the soluble form, since a part of this remains attached to the insoluble, and can only be separated from it by repeated flocculations from neutral or alkaline solution. Probably it is enclosed within the particles, from which it is only set free by repeated complete dispersions in water.

If the insoluble fraction, freed as above from the soluble product, be dissolved in water, and flocculated in an electric field, it separates out readily as a bulky flocculum. The nitrogen remaining in the supernatant fluid, however, even after repeated flocculations, is appreciably higher than that due to the actual solubility of this fraction when freed from the soluble ana-gelatin (*i.e.* about 3 mg. nitrogen per 100 cc.). (See Experimental Details, Series C.) The insoluble product was also converted into the sodium salt and into the hydrochloride, and separated again from the solution of these salts by means of electrolysis. In both cases it was precipitated in the form of a flocculum. When separated from the solution of the hydrochloride, the nitrogen remaining in the supernatant liquid was lower—when separated from the sodium salt, higher—than the nitrogen remaining in the supernatant fluid when the unionised product was flocculated. In all cases, however, it was higher than 3 mg. per 100 cc. (See Experimental Details, Series A, Part 2.)

These facts seem to the authors to have a definite significance. In an earlier paper of this series [Knaggs, Manning and Schryver, 1923] it was found that gelatin after being freed from inorganic salts readily separated from dilute solution as a flocculum. After repeated separation from solution ("recrystallisation") the nitrogen in the supernatant fluid became constant, and it was assumed at the time that a product had been obtained which was in equilibrium with its saturated solution. Certain facts, however, militated against this assumption. It was found that, if a solution were prepared containing enough gelatin for saturation and this were concentrated at low temperatures to a small bulk, the gelatin did not always separate, as it would have been expected to do from a supersaturated solution. This was especially the case if the saturated solution had been allowed to stand a day or two before concentration.

These facts indicate that "insoluble" gelatin cannot be dispersed in water at 25–30° without undergoing a certain amount of change and of conversion to the soluble form, and Manning [1924] showed that the most highly purified gelatin obtained up to the time of his work underwent a distinct irreversible change at a slightly higher temperature (37°) which was readily revealed by viscosity measurements. In the experiments described in detail later (Series A, Part 2 and Series C) it would appear that, on dispersion in water at 30°, from 3–5 % of the insoluble ana-gelatin is converted into a soluble non-flocculable form.

The soluble ana-fraction.

This was obtained by concentration of the supernatant fluid and washings from the anionic flocculation at room temperature. No gel was obtained, but a thick syrupy solution, from which the protein could be obtained by means of acetone as a white granular powder. (See Experimental Details, Series D.) This is a substance of high molecular weight, 50,000–60,000, which appears to vary slightly with the concentration [cf. Horne, 1924]. (See Experimental Details, Series E.)

The protein is readily precipitated from the syrup by saturated ammonium sulphate. Precipitation commences at 28·5 % saturation, and is complete at or about 50 % saturation. Its solution normally possesses a p_H of 4·8, which is probably its isoelectric point, whereas the p_H of the suspension of the insoluble product was 5·8. The latter could be dried in the form of sheets, not distinguishable at first sight from those of ordinary gelatin. (See Experimental Details, Series F.)

Attempt to resynthesise a gel from soluble and insoluble ana-gelatins.

It was thought that it might be possible to obtain a gel by recombination of the soluble and insoluble ana-gelatins, since neither fraction alone was in the form of a gel. These were accordingly mixed in the proportions in which they were obtained by the electrolysis of their sodium salts as above, and the solution of the mixture was dried down into a sheet at room temperature. The sheet was redissolved in water, and to the solution hydrochloric acid was added to bring it to p_H 3·0. The gelatin was then separated cationically by electrolysis. It did not, however, come down in the form of a gel, but as a flocculum. The supernatant fluid, furthermore, contained the same amount of nitrogen as would have been found in that from (*a*) the electrolysis of the hydrochloride of the insoluble fraction alone, plus (*b*) the nitrogen of the added amount of soluble fraction. There is no evidence, therefore, that the soluble and insoluble ana-gelatins can be made to recombine to form a gel. Now certain other facts have been discovered, which offer an explanation of this negative result.

It was found that the cata-gelatin gel, obtained by the electrolysis of the hydrochloride, gave on hydrolysis a diamino-nitrogen value of about 29. The corresponding number for the insoluble ana-gelatin was 23·5, while that for the soluble ana-gelatin was found to be 19. These numbers indicate, therefore, that the molecules in the ana- and cata-gelatins possess a different structural arrangement, and thus the cata-gelatin gel is not a simple combination of the soluble and insoluble ana-fractions. (See Experimental Details, Series H.)

THE RELATIONSHIP OF THE SEPARATED PROTEINS TO ONE ANOTHER, AND THE MECHANISM OF GEL FORMATION.

Proteins yield on hydrolysis in many cases single amino-acids in very small amounts. If these are formed from a single kind of molecule, or from aggregates of the same kind of molecules, the latter must be of very large size. It is conceivable, however, that proteins may be made up of aggregates of different kinds of molecules, each with a skeletal polypeptide structure, held together in some form of loose combination. Many attempts, which need not be discussed here in detail, were made to separate pure gelatin into such constituents, by its submission, after various treatments, to the action of an electric field. It is possible that by the electrolysis of the sodium salts as described the scission of gelatin has been effected into constituent proteins (which may have undergone intramolecular change during the process) which have no known relationship to one another, beyond their association together to form the gelatin complex.

There is, however, another possible explanation of the results, which needs discussion, according to which the two proteins obtained by the electrolysis of sodium gelatinate are much more intimately related to one another. Before dealing with this possibility, however, it is necessary to digress for a moment in order to discuss the effect of heating gelatin with water.

When gelatin is warmed with water, it undergoes an irreversible change, and some experiments, quoted above, indicate that this takes place to a small extent when a flocculated gelatin is dissolved, or rather dispersed, in water. Hitherto, it has been generally assumed that this change is of a degradative nature, accompanied by the scission of some of the polypeptide linkages. Some colour is given to this assumption by experiments recorded by various authors, and by the fact that commercial preparations of gelatin contain simple nitrogenous constituents which readily pass through the membranes used for dialysis. A change into simpler nitrogenous products readily takes place when the gelatin samples have not been completely freed from salts, and also occurs during its preparation on an industrial scale from its precursors, which, before the extraction process, have been submitted to the action of fairly strong acids or alkalis, which are not completely removed by subsequent washing with water. (See Experimental Details, Series A, Part 2.)

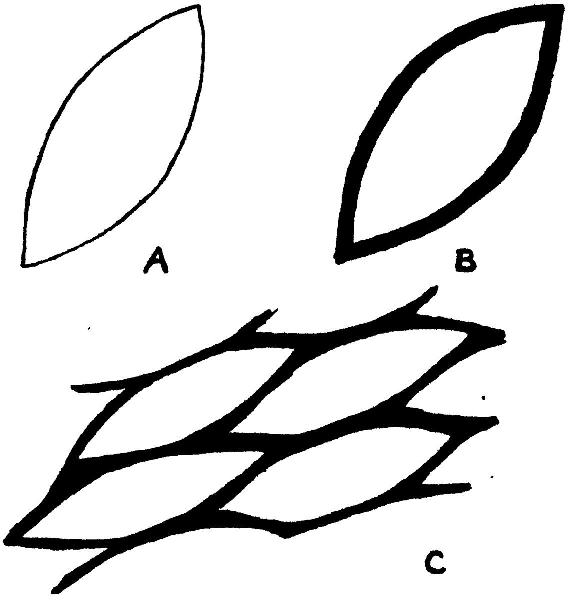
Now experiments carried out in this laboratory on an ash-free gelatin, which had been separated from simple nitrogenous products by flocculation in an electric field followed by thorough washing of the flocculum, indicate that the irreversible change produced by heating with water is not of the degradative character mentioned. The authors are indebted to Dr A. B. P. Page for these experiments. (See Experimental Details, Series K.)

It was found that in the case of a 2 % solution of a purified gelatin which had been boiled for 22 hours under a condenser—a treatment which produces a profound change in its properties—no evidence of peptide scission could be detected, either by the Foreman method of titration in alcohol, or by Van Slyke's nitrous acid method. When, however, Sørensen's formaldehyde method was employed, and the titration was carried out electrometrically, it was found that the heated gelatin solution required a very small amount more alkali to bring the E.M.F. to 650 millivolts than did the unheated portion. If this slight excess required be ascribed to the scission of peptide linkages, then the nitrogen involved in this process amounts to distinctly less than 1 % of the total, and it is extremely improbable that this just detectable change can account for the marked changes in the properties (such as solubility, flocculability, etc.) of the solution.

A similar conclusion was arrived at by an entirely different series of experiments. A gelatin solution which had been boiled for 5 hours (during which period the greater part of the viscosity change had taken place) was submitted to fractional dialysis. This was carried out in hollowed ebonite cylinders, which were clamped together at their flattened edges, between which was secured a collodion membrane, which thus separated the two cylinders. Into one cylinder was introduced the heated gelatin solution, and into the other water. Arrangements were made to secure a difference between the pressures on the two sides of the membrane, to accelerate the rate of dialysis and prevent dilution of the gelatin solution. A membrane of small permeability, softened by the method of Brown [1922] in 60 % alcohol, was first used, and dialysis was continued until no further nitrogen could be detected in the dialysate. The membrane was then replaced by a more permeable one (soaked in 80 % alcohol). After completion of a second dialysis, the heated gelatin solution was submitted to a third, using a membrane soaked in 90 % alcohol, the most permeable collodion membrane which can be manipulated without difficulty. As a result it was found that only minute traces of nitrogen passed the membranes, even when the most permeable one was employed. It appears, therefore, as a result of these experiments, that the irreversible change in gelatin produced by the action of water is not due to peptide scission, but to the change into a soluble, non-gelling, complex protein.

In the literature the term "meta-protein" has been suggested for natural proteins which have undergone some change during "denaturation," such as coagulated egg-albumin. This term may be conveniently retained to include products which have been obtained from proteins without measurable peptide scission. The products obtained by heating with water a purified gelatin may therefore be designated "meta-gelatins."

To revert now to the discussion of the explanation of the relationship between the two protein fractions, soluble and insoluble, of gelatin. Evidence has been given which indicates that a certain amount of meta-gelatin is produced when a flocculated gelatin is dispersed in water at 30°. If one assumes the micellar structure for gelatin, then it is conceivable that during the dispersal process, the peripheral part of the micellar structure undergoes change into the soluble meta-gelatin. When the changed micellae are sufficiently close together, the outer layers of meta-gelatin could form a continuous phase and thus produce a gel, as shown in the diagram.



A. Micella in original flocculum.
B. Micella surrounded by meta-gelatin.
C. Gel.

On cooling dilute solutions, however, the unchanged gelatin would separate as a flocculum, whereas the greater part of the meta-gelatin would remain dissolved in the water-phase, a small amount being also carried down with the flocculum. If this view is correct, a more complete separation of the gelatin and meta-gelatin takes place after electrolysis of the sodium salts than after electrolysis of the acid or neutral solution. The products provisionally called insoluble and soluble ana-gelatin would therefore be simply gelatin and meta-gelatin, which have undergone intramolecular change during the electrolysis of the solution of their sodium salts[1]. In the cata-form, the soluble and insoluble fractions appear to be in some form of combination, possibly due to the presence of the free amino-group of *dl*-lysine in the insoluble fraction (*vide supra*, p. 1284) and the somewhat acidic character of the soluble meta-gelatin.

The data at the moment available are not sufficient for determining which of the above explanations of the relationship of soluble ana-gelatin to insoluble ana-gelatin is correct. By the employment of the experimental methods described above, it may, however, be possible to arrive at a definite solution of the problem. By heating gelatin with water for a relatively short time, it is possible to convert a small part of the gelatin into meta-gelatin. The two fractions could then be separated either by flocculation in an electric field or by electrolysis of their salts. The floccula or gels thus obtained could then be heated again and a further fraction of soluble product obtained. This process could be repeated until no more insoluble fraction remains. By systematic investigation of the soluble fractions it should be possible to ascertain whether gelatin is made up of several different proteins, or whether the insoluble gelatin consists mainly of one class of molecules, which by the action of hot water are converted by stages into the soluble form. Work on these lines is now proceeding.

The authors, before concluding, would like again to draw attention to the importance of considering the intramolecular changes which take place so readily when gelatin is submitted to mild treatment. They are of the opinion that the ordinary physico-chemical methods, the results of which have been interpreted on the assumption that gelatin is simply a colloidal ampholyte, are not, by themselves, sufficient to explain the various properties, including gel formation, of this product.

EXPERIMENTAL DETAILS.

Series A. Part 1.

Cationic flocculation. 3·5 g. of gelatin, freed from ash by washing and electrodialysis and freshly flocculated from 1·5 % neutral solution, were washed on the filter and made up to 1·5 % solution by warming at 40°. The solution was brought to p_H 3·0 with HCl and submitted to a potential difference of 200 volts in the apparatus previously described. After 6 days no further acid could be detected in the anode liquor and the current passing was 0·2 milliamp. The nitrogen in the solution was found to vary from a very low value at the top to 4·4 mg. per 100 cc. towards the bottom of the vessel. The bulk of the gelatin was precipitated as a hard clear gel on the lower membrane, about 1″ thick. This cake was broken up and the solid filtered off.

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[1] The view that gel formation is due to the presence of soluble and insoluble products is, of course, not new, though the above method of presentation probably is. The view has recently been subjected to experimental investigation by Northrop and Kunitz [1926]. They claim to have partially separated gelatin, purified by Loeb's method, into fractions differing in their solubilities in water. The authors of this paper are of opinion that the methods adopted by Northrop and Kunitz are open to criticism on various grounds. In the first place, their own experiments indicate that a gelatin, purified by Loeb's method, still contains sufficient degradation products to account for almost the whole of their soluble fraction. (See also Experimental Details, Series A, Part 2.) Secondly, the repeated solution of their precipitates of the more insoluble fraction in water at 32° must result in the partial conversion of the more insoluble fraction of the gelatin into the more soluble. It is probable that the two fractions obtained by these workers represent two different mixtures of soluble and insoluble gelatin, more or less contaminated with degradation products. A much more convenient method of separating gelatin into soluble and insoluble fractions has been described in previous papers of this series, which have been completely ignored by Northrop and Kunitz.

N in supernatant liquid (200 cc.)	6·8 mg.	Total soluble N = 13·1 mg.
N in filtrate from broken solid (30 cc.) ...	6·3 mg.	
N in original solution	380·0 mg.	
Hence N not precipitated	3·5 % of total N	

Anionic flocculation. A similarly prepared solution was brought to p_H 8·0 with KOH, by titration. An apparatus completely enclosed was used in order to avoid absorption of CO_2 from the air by the alkaline solution, freshly boiled distilled water being employed throughout for the same purpose. After 24 hours under a P.D. of 200 volts a bulky white flocculum was precipitated, and after 4 days no further alkali could be detected in the cathode liquor. The flocculum was filtered off and the nitrogen determined in the solution.

N in supernatant liquid (125 cc.) ...	29·0 mg.	Total soluble N = 54·6 mg.
N in filtrate from flocculum (100 cc.)	25·6 mg.	
N in original solution	310·5 mg.	
Hence N not precipitated	17·7 % of total N	

Thus from the acid solution almost the whole of the gelatin is obtained as a clear gel, while from the alkaline solution a considerable portion of the nitrogen remains unprecipitated and the rest is obtained as a flocculum.

Series A. Part 2.

Flocculations of crude gelatin. A series of flocculations was carried out on samples of unpurified gelatin with a view to determining whether (*a*) separation into two fractions by anionic flocculation, and (*b*) precipitation as gel by cationic flocculation took place. The proportion of nitrogen of true soluble protein to the nitrogen merely due to soluble breakdown products was also investigated.

A sample of gelatin which had been freed from ash by washing and electrodialysis, but not by flocculation, was separated by electrolysis from acid (p_H 3), alkaline (p_H 9), and neutral solutions; the results after 5 days are given in Table I.

Table I. *Unflocculated ash-free gelatin.*

p_H of original solution (colorimetric measurement)	Type of precipitate obtained	Total N in solution mg. per 100 cc.
5·0	Bulky flocculum	36·4
3·0	Gel + slight flocculum	33·6
9·0	Flocculum (less bulky)	39·7

Thus, using the crude material, a gel may be obtained by means of cationic flocculation, but the soluble nitrogen is high in all cases. Further experiments show that this is due to the presence of diffusible degradation products.

Some Coignet's "Gold Label" gelatin was flocculated directly from a 1·5 % solution at p_H 3·4, the outside of both membranes being washed very completely with a stream of freshly boiled water. After 4 days a clear gel was precipitated and after 9 days the current had fallen nearly to zero. The thick layer of approximately 10 % gelatin gel was removed and the N determined in the supernatant and wash-liquids.

Table II. *Coignet's "Gold Label" gelatin.*

	mg.	% of total N
Total N in supernatant liquid ...	44·7	7·15
Total N in outer liquid at cathode	9·4	1·50
Total N in outer liquid at anode	19·3	3·09
Hence total soluble N	73·4	11·74
And total diffusible soluble N ...	—	4·59

Thus, in the case of crude gelatin, the true soluble ana-gelatin is accompanied by an amount of diffusible degradation products, the proportion of which approximates to 4·6 % [cf. Northrop and Kunitz, 1926]. These substances remain in solution equally when the gelatin is precipitated anionically or cationically, the slight differences observed above being due to the soluble ana-gelatin itself. After flocculation, however, the bulk of the nitrogen remaining in solution is due to the soluble fraction, the diffusible products representing only 0·5 % of the total. Further flocculations reduce this to zero.

The gel obtained from flocculation of the crude gelatin was reflocculated from acid and alkali; the results (Table III), which are typical, show the reduction in the diffusible nitrogen effected by the second flocculation, and also the difference between the quantities of soluble nitrogen in the case of anionic and cationic flocculations.

Table III.

	Reflocculation of gelatin from alkaline solution			Reflocculation of gelatin from acid solution		
	mg. N per 100 cc.	Total N mg.	N %	mg. N per 100 cc.	Total N mg.	N %
N in supernatant liquid and filtrate	18·7	44·88	7·51	11·2	25·76	4·48
N in outer liquid at anode ...	—	0·95	0·38	—	1·60	0·64
N in outer liquid at cathode ...	–	1·08	0·43	—	Trace	—
Total diffusible N	—	2·03	0·81	—	1·60	0·64

Further flocculations of purified gelatin. Similar results were obtained from the large-scale experiments in which the soluble ana-gelatin was prepared. The freshly washed flocculum, corresponding to 36 g. of gelatin, was dissolved at 37°, and made up to 2½ litres. The solution was brought to p_H 9·0, and then electrolysed for 5 days, the current eventually falling to about 1 milliampere. Both poles were kept washed and the liquids afterwards acidified and evaporated down.

Table IV.

	mg. N	% of total N
Total N (approx.)	6000·0	—
N passing anode membrane	19·4	0·36
N passing cathode membrane	9·7	0·16
N in supernatant liquid ...	799·3	13·32

A part of the 0·52 % diffusible nitrogen obtained here and in other experiments on purified gelatins is probably due to the membranes, which are usually found to be slightly permeable at the commencement of an experiment. Lower values are obtained with older membranes.

An additional experiment may be quoted here to illustrate the effect of the p_H on the result of the flocculation. The gelatin used had been freed partially from the soluble fraction by flocculating twice from neutral 1·5 % solution. It was thoroughly washed on the filter, pressed and reflocculated from neutral, acid, and alkaline solutions by redissolving as quickly as possible at about 30° and instantly electrolysing. The nitrogen in the total liquid was determined after 5 days, the precipitate being centrifuged off in the usual way.

Table V.

Original p_H of solution	Total N mg. per 100 cc.	Type of precipitate	N in solution mg. per 100 cc.
5·0	127·2	Bulky flocculum	9·8
3·0	140·0	Clear weak gel	7·4
9·0	146·1	Flocculum (less bulky)	11·3

Thus cationic precipitation seems to cause the two separate fractions to remain held together, whereas in alkaline solutions they tend to separate. Neutral solutions occupy an intermediate position. If now from the values for nitrogen in solution, given above, be subtracted an amount representing the actual solubility of the insoluble fraction the determination of which is given below (Series B), 4·4 mg. per 100 cc., or 3·5 % of the total nitrogen, is found to be the smallest percentage of nitrogen converted to the soluble form by the act of dispersing in water and warming. The neutral and alkaline solutions yield values of 4·9 and 5·7 % respectively.

B.

Solubility of the "insoluble" fraction. The residual flocculum from the anionic flocculation described under Series D, representing 87 % of the gelatin, was pressed on the filter and washed thoroughly to be as free as possible from the soluble ana-gelatin.

10 g. of the flocculum (7·5 % gelatin) were shaken for 45 minutes with 10 times the volume (100 cc.) of ice-cold freshly boiled distilled water. The whole mass was centrifuged and the slightly opalescent liquid filtered and the nitrogen determined therein. The residual substance, still containing small lumps unbroken by the shaking, was again shaken with 10 volumes of water and centrifuged, and this process was repeated until an approximately constant value for the nitrogen extracted on each shaking was reached.

Table VI. *Soluble N extracted by shaking with cold water.*

First shaking	23·8 mg. N per 100 cc.
Second ,,	14·0 ,,
Third ,,	3·6 ,,
Fourth ,,	2·9 ,,
Fifth ,,	3·4 ,,
Sixth ,,	2·5 ,,

Apart from slight irregularities due to the difficulty of completely removing the finely suspended solid from the clear solution, these figures show an approximately constant solubility, after three extractions with water, of 2·5–3·0 mg. N per 100 cc., corresponding to 15 parts of gelatin in 100,000.

Series C.

Further flocculation of the insoluble fraction (*ana-gelatin*). The previous determination of the solubility of gelatin [Knaggs, Manning and Schryver, 1923; Manning, 1924] was based on the nitrogen remaining in solution in the supernatant fluid after repeated flocculations. This figure, 48 parts per 100,000, cannot be regarded as accurate, since the work recorded in the previous section shows that the bulk of the soluble nitrogen is in the liquid immediately surrounding the flocculum, and not diffused equally throughout the supernatant fluid. The following determinations were therefore made on the whole liquid.

The insoluble residue after six extractions with water was warmed to 40° for 2 minutes in 30 cc. water, and cooled instantly. The opaque liquid was at once flocculated. Within 30 minutes a precipitate had formed, and after 2 days' electrolysis the heavy white flocculum was centrifuged off and the nitrogen determined in the filtered liquid.

Nitrogen in solution after flocculation 13·2 mg. per 100 cc.

By repeated flocculation, however, the soluble nitrogen can be reduced to a lower limit than the above. Thus 11 g. flocculum were made up to 33 cc. in water at 37° and flocculated repeatedly. A more and more bulky flocculum was obtained each time, and eventually a constant value for the soluble nitrogen was obtained.

Table VII.

	Time of flocculation (hours)	Soluble N mg. per 100 cc.
First flocculation	48	26·3
Second ,,	96	11·2
Third ,,	48	12·2
Fourth ,,	96	8·8
Fifth ,,	48	8·1

Longer periods of flocculation lead to the attainment of the equilibrium in fewer operations.

Table VIII.

	Time of flocculation	Soluble N mg. per 100 cc.
First flocculation	48 hours	22·6
Second ,,	7 days	14·4
Third ,,	5 ,,	6·8
Fourth ,,	3 ,,	6·8

These numbers are only about one-half that for the nitrogen left in solution after flocculating the material which had been thoroughly extracted with water, and this fact seems to indicate that a part of the soluble fraction is enclosed within the micellae, is not removed by extraction with water and can only be set free on dispersion.

This smallest number, 6·8 mg. per 100 cc., is greater than that obtained by the simple extraction with water of the washed flocculum. If the latter number (2·5 mg. per 100 cc.) be subtracted from the former, a figure should be obtained which would give a measure of the amount of insoluble gelatin converted to the soluble form (*vide supra*, p. 1287) on dispersing in water at 37°, namely, 4·3 mg. per 100 cc., or 3·7 % of the total nitrogen. Reference may be made to experiments at the end of Series A, Part 2, which confirm approximately this calculation, the value there given being determined for 30°, and therefore slightly lower than the above.

The very slight irregularities in the final numbers may be ascribed to:

(*a*) different times of flocculation;

(*b*) variation in the resistance of the membranes and of the whole system, and therefore of the current density applied;

(*c*) temperature effects, the outside temperature varying over a range of 5°.

Series D.

Isolation of the soluble fraction. 45 g. of purified gelatin, freed from ash, were flocculated from water once, and the white flocculum filtered and washed with water. Three litres of 1·3 % solution were then made up by warming and brought to p_H 9·0 with KOH, 0·4047 g. being required. This solution was then flocculated, the apparatus being enclosed and the cathode being kept washed to remove alkali. The voltage was slowly raised from 50 to 200 volts, as the resistance of the solution increased, an average current of about 4·5 milliamperes being passed. After 10 days no further alkali could be detected in the cathode washings; the flocculation was therefore stopped, the clear supernatant liquid removed as completely as possible, and the flocculum filtered on paper and washed. The supernatant liquid and filtrate combined showed a nitrogen content of 16·1 mg. per 100 cc., or a total of 480 mg. of soluble nitrogen. Washings from the precipitate contained a further 17 mg. nitrogen. The bulky precipitate was pressed on a Büchner funnel to remove as much as possible of the soluble fraction adhering, and the filtrate liquids, totalling 2·9 litres, were then evaporated to 60 cc. at ordinary temperature, by means of a fan. The resulting clear syrup was poured slowly into 8 volumes (500 cc.) of absolute acetone, stirred thoroughly, and after standing overnight, stirred, filtered, stirred up with a further small quantity of acetone and again filtered. This process was repeated three times. Finally, the precipitate was sucked dry, transferred to a porous tile and dried *in vacuo* for several days.

A dull white granular powder was thus obtained; it was only very slightly hygroscopic when thoroughly dried, and dissolved in water to a clear pale yellow solution, without warming. It charred on heating, without melting. It was completely precipitated by ammonium sulphate solution, the precipitation limits being between 28·5 and 50 % saturation.

Series E.

Osmotic pressure determinations. Sac membranes of necolloidine were prepared, and soaked in 60 % aqueous alcohol before use [Brown, 1922]. A rapid rise was observed, reaching a maximum in 1–3 days, and afterwards a slow fall. If started above the constant value, a rapid fall was observed, reaching the constant value and remaining there for some days, followed by a slight further fall.

Table IX.

N in final solution mg. per 100 cc.	Constant osmotic pressure (mm.)	Temperature ° C.	Molecular weight
223·2	64·0	20·5	53,300
287·2	76·0	18·5	57,400
302·4	74·5	17·0	61,600

This molecular weight, although varying slightly with concentration [cf. Horne, 1924], is definitely that of a complex protein.

Series F.

Hydrogen ion concentration determinations. In general, solutions of soluble ana-gelatin are more acidic than those of the insoluble portion. The p_H of the syrup from the anionic flocculation was found to be 4·1 and 4·2 respectively, in two preparations. These may have been too acid owing to traces of CO_2 in solution. A quantity of the syrup, diluted with three-quarters of its volume of boiled water, was therefore electrolysed for 3 days till all traces of electrolyte were removed; the resultant solution had a p_H of 4·8. This probably represents the isoelectric point of the soluble ana-gelatin.

The electrometric buffer-titration curve is fairly smooth. It shows a slight point of inflection at p_H 4·8, but no obvious inflection at p_H 7·7 [cf. Wilson and Kern, 1922, 1923].

The p_H of solutions of insoluble ana-gelatin varied from 5·6 to 6·8, and of the flocculum itself from 5·1 to 6·5. A quantity of the substance, freshly flocculated from alkaline solution, was mixed with water without dissolving and electrolysed for 3 days till all traces of electrolyte were removed. The p_H of the precipitate, determined colorimetrically by spreading out on a watch-glass with the indicator, was then 5·8, and of the supernatant liquid 6·1. Probably 5·8 represents the fairest value of the isoelectric point of insoluble ana-gelatin.

It should be noted, parenthetically, that the p_H of the solution after electrolysis of a crude gelatin corresponds with that of the soluble fraction, which is present in large amount, while the p_H of the solution after electrolysis of a purified sample approximates to 5·8, the isoelectric point of the insoluble fraction.

Series G.

Attempt to resynthesise a gel from soluble and insoluble ana-gelatins. To a quantity of the insoluble ana-gelatin some of the syrupy solution of the soluble fraction was added, in such proportion that the added nitrogen was equivalent to 12 % of the total nitrogen in the mixture, this corresponding to the proportion of the soluble ana-gelatin present after anionic separation. The mixture was melted at 37°, and on cooling a fairly clear gel was produced, which was cut up and dried down in the cold, redissolved, made up to p_H 3·0, and flocculated. After 6 days the current had fallen to 0·5 milliampere and the p_H of the anode liquid was 4·5. The compact flocculum obtained was centrifuged off. The nitrogen in solution showed no recombination.

Table X.

Soluble N calculated	mg. per 100 cc.
Due to added soluble ana-gelatin	13·5
Due to insoluble ana-gelatin, corresponding to second flocculation (see Series C)	14·4
Total	27·9
Soluble N found	30·4

The agreement is sufficiently close to leave no doubt that the two fractions, once separated, do not recombine to form a gel.

Series H.

Hausmann numbers of the fractions.

Soluble ana-gelatin. The nitrogen distribution was determined by the micromethod previously described [Thimann, 1926] both on the concentrated syrup before precipitation and on the dried and powdered soluble substance. Close agreement was observed, as the following numbers show, so that the precipitation by means of acetone does not appreciably affect the product. Furthermore, standing with acid in the cold before hydrolysis causes no increase in the basic nitrogen [cf. Knaggs, 1923]. The third set of numbers in the table was obtained after allowing the powder to stand for 24 hours in 18 % HCl.

Table XI.

	Amide-N	Humin-N	Diamino-N	Mono- + non-amino-N	Total N
Syrup	2·8	0	19·2	77·3	99·4
Powder	3·3	Trace	19·3	77·5	100·1
Powder (stood in acid)	3·3	0	18·3	78·4	100·1

In each case these represent the mean of at least two determinations.

Insoluble gelatins. The original flocculum from the anionic flocculation contains still some soluble fraction, as was shown above, but this amounts only to a small percentage of the total, and hence should not greatly affect the hydrolysis numbers. A slight increase in the amide-nitrogen of the unwashed gelatin may be due to the soluble fraction present. The catagelatin gel shows, however, a higher figure than either, pointing to structural difference. The insoluble ana-gelatin is more labile than the soluble, since on standing in 18 % acid before hydrolysis, the basic nitrogen is definitely increased.

Table XII.

	Amide-N	Humin-N	Diamino-N	Mono- + non-amino-N	Total N
Precipitate from flocculation in alkaline solution	1·8	0	23·2	75·3	100·3
Ditto, after six washings	1·6	0·1	23·5	74·9	100·1
Ditto, after standing 24 hours in 18 % HCl	1·5	0	25·9	72·7	100·1
Cata-gelatin gel	1·5	0	29·2	69·2	99·9

Two differences are thus observed between the soluble and insoluble ana-fractions; the amide-nitrogen, which is nearly twice as high in the former case, and the diamino-nitrogen, which is 4 % higher in the latter. The insoluble cata-gelatin has the same amide-nitrogen as the insoluble ana-gelatin, but a higher diamino-nitrogen than either of the ana-gelatins.

(In experiments upon the effect of acid and alkali treatment of the precursor of gelatin, it was found that the diamino-nitrogen of the precipitated portion, after alkali treatment, was 24·7, and of the supernatant liquid (corresponding with the soluble fraction) about 18 %. These numbers show close agreement with those above, which may thus give a clue to the effect of precursor treatment [Knaggs and Schryver, 1924, 3].)

Series K. (Experiments by Dr A. B. P. Page.)

Effect of heat treatment of purified gelatin. Within the somewhat large limits of error in the Van Slyke method for determination of amino-nitrogen there was no change in the amino-nitrogen after heating for 20 hours at 100°.

Electrometric formaldehyde titration. The heated gelatin was diluted 1 : 1 before titration. Titrations were then made until the E.M.F., using a hydrogen electrode, reached 650 millivolts—the limit beyond which the electrodes became unreliable in the presence of formalin.

Titre of the 25 cc. formalin	2·95 cc. NaOH		
Titre of 25 cc. unheated 2 % gelatin	9·25 − 2·95 =	6·3	cc. NaOH
Titre of 25 cc. unheated 1 % gelatin		3·13	,,
Titre of 25 cc. heated 1 % gelatin	6·50 − 2·95 =	3·55	,,
Hence difference due to heating		0·42	,,
This is equivalent to		0·29	mg. nitrogen
25 cc. heated 1 % gelatin contained		42·0	,,
Hence % increase of amino-N on heating a 2 % solution for 21 hours at 100°		0·69 %	

Fractional dialysis. 300 cc. of a 1·85 % solution of a once flocculated gelatin were heated for 5 hours at 100°. 200 cc. were used for dialysis. The collodion membranes were poured on a glass plate from 5 % alcohol-ether solution of necolloidine, and covered with a Petri dish. They were air-dried, soaked overnight in alcohol of the required strength and finally washed for 3 or 4 hours in frequent changes of distilled water.

Two or three changes of dialysis water were made with each membrane, and the nitrogen determined in each. The amount of nitrogen which diffused in the time, and its percentage of the total nitrogen are given below.

Table XIII.

Membrane soaked in	Time of dialysis (hours)	Total N diffused (mg.)	% of total N
60 % alcohol	21	0·39	0·06
	Subsequent 67	0	0
	„ 89	0	0
	Total 177	0·39	0·06
80 % alcohol	16	2·0	0·32
	Subsequent 40	0·5	0·08
	„ 84	0·4	0·06
	Total 140	2·9	0·46
90 % alcohol	25	0·75	0·12
	Subsequent 41	0	0
	Total 66	0·75	0·12

Hence the total nitrogen diffused in 383 hours = 0·64 %. This agrees with the change in amino-nitrogen, and also with the diffusible nitrogen found for purified gelatin in Series A, Part 2.

SUMMARY.

1. Evidence has been given to show that the molecules, or aggregates of molecules, of which gelatin is composed readily undergo intramolecular change.

2. This evidence suggested the possibility that the intramolecular arrangement was different according to whether gelatin was functioning as anion or cation.

3. By the electrolysis of the hydrochloride, gelatin was obtained in the form of a gel, with very little nitrogen in the supernatant fluid. By the electrolysis of the sodium salt, two fractions were obtained, *viz.* an insoluble fraction (insol. ana-gelatin) and a soluble fraction (sol. ana-gelatin). Both were complex proteins. Insoluble ana-gelatin is soluble in water to the extent of about 15 parts in 100,000 at 15°; soluble ana-gelatin has a molecular weight of 50,000–60,000.

4. Attempts were made to resynthesise a gel by combination of the soluble and insoluble ana-gelatins, but without success.

5. Two possible relationships between the soluble and insoluble proteins into which gelatin is separated on electrolysis of the sodium salt are suggested:

(*a*) that they are independent proteins having no relationship to one another other than their association together in the gelatin complex;

(*b*) that the soluble fraction is a product of the action of water on an insoluble gelatin. The combination of a soluble and insoluble fraction in some form is responsible for the gel formation.

On electrolysis of the sodium salt the two fractions separate, undergoing in the process intramolecular change, which prevents their recombination.

6. Evidence is given to show that by the action of water on gelatin a product is produced which is a highly complex protein, and that the change is not accompanied by any appreciable amount of peptide scission. For the changed gelatin the term *meta-gelatin* is suggested. If the second of the above conceptions is correct, the gel called cata-gelatin is a close combination of gelatin and meta-gelatin, which separate on electrolysis of their sodium salts, undergoing at the same time an intramolecular change. There is also evidence that insoluble gelatin cannot be dispersed in water without undergoing a slight irreversible change and conversion to a soluble form.

The authors are much indebted to Dr Mastin for help in the earlier stages of this work, and also to the Adhesives Committee of the Department of Scientific and Industrial Research, through whose agency the services of Dr Mastin were made available. One of the authors (K. V. T.) is also indebted to the Department for a studentship.

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CLXXI. INVESTIGATIONS ON GELATIN.

PART X. THE EFFECT OF PREVIOUS TREATMENT WITH ACID AND ALKALI ON THE DIAMINO-NITROGEN FRACTION OF THE HYDROLYSIS PRODUCTS OF GELATIN.

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In these experiments Coignet's Gold Label gelatin purified by electrodialysis was used. Gelatin was allowed to stand in contact with HCl solutions varying in strength from concentrated acid to *N*/70. The most convenient quantity of gelatin for determining the Hausmann numbers by the micro-method [Thimann, 1926] is 0·1 g. of the dry protein, and this amount was taken throughout. After standing for 48 hours at room temperature the solutions were hydrolysed by pouring into boiling acid at such a rate that the boiling was not interrupted. The acid in the flask was of such a strength that the final mixture was approximately 20 %.

A few duplicate experiments were performed allowing a time of 3 days before hydrolysing instead of 48 hours. As the results agreed fairly closely it was assumed that 48 hours was sufficient for equilibrium to be reached.

Table I (see also Fig. 1).

Concentration of HCl solution	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
0	2·6	17·7	79·6	99·9
0·0143 *N*	2·4	17·8	79·8	100·0
0·1 *N*	2·7	18·4	79·1	100·2
0·77 *N*	2·4	20·6	77·2	100·2
1·4 *N*	2·3	22·3	75·2	99·8
2 *N*	2·5	23·0	74·3	99·8
2·3 *N*	2·4	23·5	73·8	99·7
3·45 *N*	2·5	22·9	74·6	100·0
5·45 *N*	2·3	21·3	76·1	99·7
8·8 *N*	2·2	15·2	82·3	99·7

Effect of NaOH *solutions.*

A similar series of experiments was carried out with gelatin in the presence of alkali. As the presence of salt influences the diamino-nitrogen precipitated by phosphotungstic acid, control experiments were performed. The hydrolysing acid in these cases contained exactly the same amount of salt as the acid used in the hydrolysis of the protein solutions.

Table II.

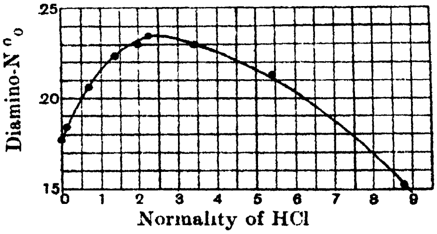
Concentration of NaOH	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
0·1 *N*	2·2	18·0	79·3	99·5
Control 0·1 *N*	2·5	18·1	78·8	99·4
N	2·8	21·0	76·7	100·5
Control *N*	2·6	18·6	78·5	99·7
2 *N*	2·6	23·6	73·4	99·6
Control 2 *N*	2·3	19·1	78·9	100·3

Hence the corrected values of diamino-nitrogen due to the influence of NaOH are as follows, the excess over 18 in the controls being subtracted from the experimental numbers:

Normality	0·1 *N*	*N*	2 *N*
Diamino-N	18·0	20·4	22·5

Effect of acid on heated gelatin.

A 10 % gelatin solution was boiled in a steamed-out Jena flask for 2 days; 1 cc. of this solution was allowed to stand with 10 % HCl solution, which is the concentration which gives the greatest rise of diamino-nitrogen with ordinary unheated gelatin.



Gelatin treated with HCl prior to hydrolysis

Fig. 1.

Table III.

	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
Heated gelatin	2·5	18·5	79·5	100·5
Treated with 10 % HCl	2·6	18·1	79·0	99·7

Effect of pepsin and trypsin.

200 cc. of 4 % gelatin solution in *N*/10 HCl were warmed to 37° and 5 cc. of 1 % pepsin solution added. This mixture was kept at 37° and at intervals 25 cc. were pipetted into a flask, cooled and titrated with *N*/10 NaOH in the presence of formalin. When the formalin titration values became constant a portion of the gelatin solution was hydrolysed and the Hausmann numbers were determined. At the same time a control experiment was set up using the same quantities of reagents but the activity of the pepsin was destroyed by boiling before it was added to the gelatin solution.

A similar experiment was performed with trypsin.

Table IV.

	Amide-N %	Diamino-N %	Mono-+ non-amino-N %	Total %
Pepsin	2·9	19·3	77·5	99·7
Control	2·4	19·0	78·2	99·6
Trypsin	2·5	20·0	77·0	99·5
Control	2·6	19·2	78·4	100·2

Hence the corrected values of diamino-N due to enzyme action are as follows:

Pepsin	18·3
Trypsin	18·8

The gelatin used had not been freed from the soluble fractions by flocculation, and therefore the numbers in this paper are not absolute; they do, however, indicate the general trend of the action of acids and alkalis on gelatin.

This work was carried out on the suggestion of Prof. S. B. Schryver.

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CLXXII. COMBINATION OF PROTEINS WITH PHTHALEIN DYES.

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Many properties of proteins have been ascribed to adsorption phenomena and surface effects—grouped under the name of colloidal properties. Loeb [1922] and others have shown that many properties of proteins may be explained by the orthodox laws of chemical combination. In this paper an attempt is made to explain the effect of proteins on phthalein dyes as a chemical phenomenon.

The interesting observation was made by Rosenthal [1926] that when a small amount of protein has been added to a dilute rose bengal solution the deep pink colour is not discharged as usual when the solution is acidified. This he ascribes to the fact that the dye behaves as though it were not in solution. It was thought that this observation deserved further attention and it was found that if the colour of a dilute rose bengal solution were discharged by acidification with hydrochloric acid and then a small amount of protein were added and the solution warmed, a rose-red colour soon developed. The combination of protein and dye resulted in colour change, so that the simple explanation of the dye behaving as though it were not in solution is hardly accurate since the same colour is obtained when the dye is undoubtedly in solution and already decolorised. The reaction between the protein and acidified dye proceeds very slowly in the cold, probably because an intramolecular change takes place as will be shown later.

The question arose as to whether rose bengal (tetraiodotetrachlorofluorescein) was unique in its behaviour or whether all fluorescein and phenolphthalein derivatives behaved similarly. The first dyes examined fell into two classes:

(I) *Colour discharge prevented by protein.*

Rose bengal, eosin (tetrabromofluorescein), erythrosin (iodofluorescein) and phloxin red (dichlorotetrabromofluorescein).

(II) *Colour discharged even when protein is present.*

Phenolphthalein, tetrachlorophenolphthalein, tetrabromophenolphthalein, phenolsulphonephthalein and bromothymol blue.

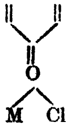
It would thus seem that fluorescein derivatives behaved in one way and phenolphthaleins and sulphonephthaleins in the other. But the deep yellow colour of alkaline fluorescein solutions changed to the pale yellow colour when acidified even when protein was present. Thus some other explanation was necessary since fluorescein behaves differently from its derivatives. It was noticed that the usual change of colour of all the dyes of class I takes place at reactions on the acid side of the isoelectric point of the protein (p_H 4·8), while the usual change of colour of dyes of class II occurs on the alkaline side of the isoelectric point. The behaviour of thymol blue (thymolsulphonephthalein) supports this observation. This dye changes from blue to yellow in alkaline solution (p_H 9·6) and from yellow to red in acid solution (p_H 2·8). When protein was added the change from blue to yellow on adding acid was not interfered with, but the change from yellow to red did not take place when small amounts of serum-proteins were present before acid was added. The p_H at which the dye changes colour, therefore, determines its behaviour.

Table I. *Reaction of colour change of dyes.*

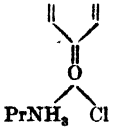
Reaction at which colour begins to change when acid is added.

Colour retained in acid protein solution		Colour discharged by acidification	
Dye	p_H	Dye	p_H
Eosin	3·0	Thymol blue—alkaline range	9·6
Erythrosin	3·6	Fluorescein	5·6
Phloxin red	3·6	Bromothymol blue	7·6
Rose bengal	4·6	Phenolsulphonephthalein ...	8·4
Bromophenol blue ...	4·6	Tetrabromophenolphthalein	9·0
Thymol blue—acid range	2·8	Tetrachlorophenolphthalein	9·4
		Phenolphthalein	10·0

From this it is evident that acid protein ($\mathrm{PrNH_3}^{\cdot} + \mathrm{Cl}'$) and coloured (quinonoid) dye must co-exist if colour discharge on acidification is to be prevented. Now Meyer and Hantzsch [1907] describe complexes of phthalein dyes and heavy metal salts (*e.g.* $SnCl_4$) which are red in colour, and therefore have the quinonoid structure, and are probably oxonium salts of the type I.



I



II

It is suggested therefore that we are dealing with dye-protein chloride compounds of the type II. Very strong support for this view is found in the fact that alkaloids similarly interfere with the colour discharge of certain phthalein dyes [Messner, 1903], as does benzidine, as pointed out to me by Dr Marrack. In the case of simple aromatic amines there can be little doubt that the mechanism is chemical combination and it seems highly probable that the same applies to proteins and dyes.

Further evidence for the view of oxonium salt formation is found in the fact that the colour change of rhodamine B is not prevented by adding protein before acidifying. Rhodamine B changes colour on the acid side of the isoelectric point of proteins (p_H 1·2) but the dye does not possess a quinonoid structure and the chromophoric group is a diethylimino-group.

Fluorescent dyestuffs no longer fluoresce when combined with protein in acid solution. When serum-albumin and acid are added to eosin and erythrosin the colour deepens, so that the acid solution is actually more deeply coloured than the original alkaline solution. In addition to this the tint is altered on adding protein and acid. The absorption spectra of the compounds were therefore studied. The author is indebted to Prof. Roaf for allowing him to use his spectrometer and calibrations.

Table II. *Absorption bands.*

Wave lengths in Ångström units.

Dye		Alkaline dye Limits	Alkaline dye Mean	Acid-protein-dye Limits	Acid-protein-dye Mean	Shift in band
Rose bengal	1st band	4978–5186	5080	5220–5360	5290	210
(0·01 %)	2nd band	5378–5522	5450	5580–5700	5640	190
Eosin (0·008 %)		5000–5270	5135	5290–5490	5390	255
Erythrosin		4980–5300	5140	5290–5480	5395	255
Phloxin	1st band	5047–5175	5111	—	—	—
	2nd band	5313–5453	5383	5443–5615	5529	146

These dyes when acidified possessed no prominent bands in the visible region of the spectrum. There is thus a shift of 150–250 Å. in the absorption bands when protein and hydrochloric acid are added to an alkaline dye. So marked an alteration in the absorption spectrum suggests the formation of a new chemical compound with properties different from its components rather than loose physical attachment or adsorption of the dye by protein micellae. One might note at this point that Rosenthal assumed that the colour of rose bengal did not change when combined with acid protein and calculated the amount of dye combined by comparison of the colour of the acid-protein dye with that of alkaline rose bengal. As there is certainly a change in tint and probably in intensity of the colour this seems hardly justifiable. His method of protein determination in the cerebrospinal fluid by this method may also be criticised on the ground that a very large difference in protein concentration has a very small effect on the colour produced.

As we have seen, closely defined conditions as to the critical reactions of the dye and protein must be observed in order that the phenomenon of colour retention in acid solution should occur. This specificity supports the view of chemical combination, rather than physical adsorption. Further support for this view is found in the shift of the absorption band which one would not expect with a purely surface effect.

If the combination of dye and protein were purely an adsorption phenomenon and surface effect it would be expected that surface-active substances, *i.e.* those lowering interfacial surface tension, would have a great effect on the combination. Ten liquids not miscible with water were chosen, viz. amyl and capryl alcohols, chloroform, ether, liquid paraffin, oleic acid, olive oil, light petroleum, toluene and turpentine. The interfacial tension of these liquids against water varies from about 4 to 40 dynes per cm. [Du Noüy, 1926], and one would expect differences in their effect on an adsorption system. It was found, however, that none of the liquids had any appreciable effect on the combination of eosin with proteins whether the liquid was shaken with the acid-protein-dye solution, or with the eosin before the protein and acid were added, except that some of the liquids extracted a small amount of acid-eosin.

Rosenthal [1925] states that 10 mg. of sodium taurocholate prevented the combination of rose bengal with one drop of serum (*i.e.* about 3 mg. of protein). Apart from the fact that this seems an excessive amount of bile salt to have to use if a pure surface effect were involved, it was found that if 10 mg. of sodium taurocholate or saponin were added to one drop of horse serum, and then 10 cc. of rose bengal and 4 cc. of $N/10$ hydrochloric acid were added, the solution remained deep pink in colour. The colour of the solution had been slightly reduced by the surface-active substance but the combination was only very partially inhibited. Possibly the result described by Rosenthal was due to the effect of the bile salt on the membranes he used. The absence of any very great effect of surface-active substances on the dye-protein combination is further evidence that the effect is not purely surface adsorption.

When considerable amounts of concentrated acids, *e.g.* 30 % hydrochloric acid, 20 % salicylsulphonic acid, are added to the acid-protein-dye solutions the colour is discharged, probably because the dye cannot exist in the coloured quinonoid form, even when combined with protein, in strong acid solution. In the case of salicylsulphonic acid the protein is, of course, precipitated, but, even when the sparse precipitate is dissolved by warming, the colour is not regained.

In all these experiments serum, serum-albumin and globulin, egg-albumin and gelatin behave similarly except that gelatin manifests less dye-combining power, possibly due to the molecular aggregation of this protein as suggested by Rosenthal [1926].

The main points supporting the view of chemical combination between the dyes and protein rather than the idea of surface adsorption are as follows.

(1) The specificity and rigidly defined conditions of p_H, etc., for combination to occur.

(2) The analogy between proteins and simple organic bases in their behaviour towards dyes.

(3) The shift in absorption band which suggests a chemical rearrangement rather than loose physical forces being called into play.

(4) The lack of effect of surface-active substances on the combination.

Small strips of silk and wool were immersed in fairly concentrated (*ca.* 1 %) alkaline solutions of the dyes, the temperature was rapidly raised to the boiling-point, the solutions were cooled and the material immediately removed from the bath, rinsed with water and immersed in $N/10$ hydrochloric acid. The results were as follows.

Dye	Silk	Wool
Eosin	Deep pink	Scarlet
Erythrosin	Magenta	Deep red
Rose bengal	Magenta	Deep magenta
Thymol blue	Yellow	Deep yellow
Phenolphthalein	Colourless	Colourless
Fluorescein	Colourless	Almost colourless

These results are thus in complete accord with those obtained with serum-proteins, egg-albumin and gelatin. Those dyes which give coloured compounds with the proteins in acid solution dye the fabrics and the dye is acid-fast, whilst dyes which do not give coloured solutions with acid-proteins do not dye the fabrics to any extent.

Similarly it was found when paraffin sections of various tissues were stained that the same tissues were stained by eosin, erythrosin and rose bengal, and the differences in tint with the different dyes were similar to those with wool and silk. When the stained sections were immersed in *N*/20 hydrochloric acid the stain was not removed. Evidently this histological protein staining is another manifestation of the chemical phenomenon described. Differential staining by treating with acids after the phthalein dyes is therefore due to the stability of the protein-dye compound in acid solution.

Rous [1925] has injected phthalein dyes into living animals and from the colour assumed by the various organs, draws important conclusions regarding the p_{H} of different tissues. The greatest caution should be exercised in drawing such conclusions since many of the phthalein dyes combine with proteins, and the tint of some of these compounds is slightly different from that of the dye itself, and the colour of the compound varies little with change of p_{H}.

It will be obvious also that many of these dyes cannot be used as indicators as the "protein error" is very great. The case of bromophenol blue may be cited as an example. In series I, one drop of the dye solution was added to 5 cc. of acetate buffer of each p_{H}. In series II, a small amount of serum was added to the dye solution, and one drop of the alkaline dye-protein solution was added to each buffer.

p_{H}	3·2	3·4	3·6	3·8	4·0	4·2	4·4	4·6
I. Dye alone ...	yellow		green-yellow	green-blue			blue-violet	
II. Protein present	green-blue			blue				

The tints of the tubes of series II are in no case the same as those of series I and are deeper, but apart from this not even an approximate idea of the p_{H} can be gained from the colour of the tubes of series II. If to each of the dye-buffer solutions of series I one drop of ten times diluted serum is added the colours alter slightly, but even after 24 hours' standing at room temperature the colours are not the same as those of series II; but if the tubes are plunged in a boiling water-bath for a few seconds and then cooled, the colours of series I and II are indistinguishable. In the tubes of series II, the dye is in the quinonoid form when brought into contact with the acid protein, hence coloured oxonium salt formation occurs immediately. In the tubes of series I, however, the acid protein is brought into contact with the dye, mainly in the phenolic form, hence combination is slow, but on warming some of the phenolic form changes into the quinonoid form and this combines with the acid protein. Change of phenolic into quinonoid dye in the cold is slow under these conditions but when warmed more quinonoid dye is formed—if a greenish solution of bromophenol blue is warmed the solution becomes blue while hot, but greenish again on cooling. The formation of some quinonoid dye from phenolic dye on heating is a property of many phthalein dyes.

The dyes bromophenol blue, rose bengal, phloxin red, erythrosin, eosin and thymol blue (the last-named in its acid range) cannot therefore be used as indicators in the presence of proteins. Since combination of the protein occurs only with the quinonoid dye, different end-points are obtained when solutions containing protein are titrated, using these dyes as indicators, according to whether acid is added to the alkaline solution or alkali to the acid solution.

In connection with these phenomena an observation of Deutsch [1927] is of interest. He observes that when an aqueous solution of thymol blue, just on the alkaline side of its range of colour-change from the yellow quinonoid to the red phenolic form, is shaken with benzene, a reddish violet colour appears, which disappears when the liquid settles down. This he ascribes to alteration of the dissociation constant of the dye when adsorbed at the benzene-water interface. There is no evidence for this assumption and another explanation is possible. Only the phenolic red form of thymol blue is soluble in benzene in which it gives a reddish violet solution. It is probable therefore that only the phenolic form is adsorbed at the benzene-water interface. In the solution used by Deutsch there is a small amount of the red form in equilibrium with a large amount of the yellow form. When the fluid is shaken the benzene-water interface is greatly increased and all the red form present is adsorbed; some of the yellow form in the aqueous solution then changes over to the red form, to maintain the equilibrium in the water, and this is promptly adsorbed and so on, the reddish colour of the dye adsorbed at the interface predominating over the yellow colour of the aqueous solution. When allowed to settle down the adsorption of the red form becomes negligible since the interface has decreased enormously, and when it passes into the water it is mostly changed to the yellow quinonoid form to give the original equilibrium. There is therefore no need to postulate a change in dissociation constant to explain the behaviour of the dye.

Up to now we have been dealing with very dilute solutions of dyes (*ca.* 0·01 %). When more concentrated solutions are employed another phenomenon has been observed. If serum-proteins are added to a fairly concentrated aqueous dye solution, *e.g.* 0·25 % eosin, and then dilute hydrochloric acid is added gradually, a reddish precipitate first forms and then, on adding more acid, the precipitate dissolves, giving a clear, deeply-coloured solution. If the dye alone is acidified a yellow precipitate is formed and this does not dissolve in excess acid. This behaviour is not confined to the dyes which remain coloured in acidified protein solutions, but occurs with all the dyes investigated, *i.e.* phenolphthalein and its derivatives, phenolsulphonephthalein and its derivatives, and fluorescein and its derivatives. Those dyes which do not remain deeply coloured in acidified protein solutions do not give deeply coloured precipitates, *e.g.* phenolphthalein gives a colourless precipitate soluble in excess acid. Serum, serum-albumin, serum-globulin, egg-albumin and gelatin all behave in the same way when sufficient protein is added. When an insufficient amount of protein is employed a clear solution in excess acid is not obtained. The precipitation of yellow phenolsulphonephthalein-serum-albumin occurred, although both the dye and the protein alone are soluble at all reactions.

Electrometric determinations were carried out with Dr J. R. Marrack's apparatus and help to determine the reactions at which precipitation and solution occurred. Precipitation commenced near the isoelectric point of the protein, *i.e.* when the protein began to function as a base, and the precipitate dissolved, giving a clear solution at about p_H 2·5. These points appeared to be independent of the nature of the dye.

	Precipitation		
Dye change-point p_H	Began p_H	Cleared p_H	Clear p_H
3 eosin—serum-albumin	4·9	2·9	2·3
3 eosin—gelatin	4·7	—	—
8 phenol red—serum-albumin	4·5	—	2·6

In three cases analysis of the protein-dye precipitates was conducted as follows: The protein was added to the alkaline dye solution and acid was added until a copious precipitate formed. This was allowed to stand overnight and was then filtered off on a hard filter-paper on a Büchner funnel. The dye precipitate was then dissolved in $N/10$ hydrochloric acid, the protein determined by Kjeldahl's method and the dye determined by adding 1 cc. of the solution to hot alcohol, making up the volume to 25 cc. and then comparing the colour of the alcoholic solution, when made alkaline, with that of standard alcoholic solutions of the dyes.

Constituents	Dye %	Protein %	$\frac{\text{Mols. dye}}{\text{Mols. protein}}$
Eosin—serum-albumin	0·27	0·85	20 : 1
Rose bengal—serum-albumin	1·765	2·26	34 : 1
Phenol red—egg-albumin*	1·1	4·66	28 : 1

* Assuming the molecular weight of egg-albumin to be 44,000 [Marrack and Hewitt].

It seems probable that these precipitates are due to salt formation between the dyes which have acidic properties and the proteins which on the acid side of the isoelectric point behave as bases. Chapman, Greenberg and Schmidt [1927] describe the formation of similar insoluble protein-azine-dye precipitates. The varying ratio of dye molecules to protein molecules is due to the fact that at these reactions the maximum acid-binding power of the protein is not reached. The solution of the protein-dye compound in excess acid is probably due to the combination of the basic groups of proteins, which are not already combined with dye, with hydrochloric acid forming soluble hydrochlorides. In support of this is the observation that when excess dye is present and nearly all the basic groups are combined with dye the compound does not dissolve in excess acid.

Since the acidic groups of the dyes at these reactions are mainly phenolic it would be anticipated that phenol itself would behave similarly to the dyes, and this was found to be the case. When a small amount of protein is added to a dilute solution of phenol and acid is gradually added, a precipitate first forms and this dissolves up when more acid is added. A typical experiment with acetate buffers was as follows:

p_H	5·2	5·0	4·8	4·6	4·4	4·2
Precipitation	—	±	+ + +	+ +	+ +	–

Hoffman and Gortner [1925] suggest that on the acid side of p_H 2·5 proteins do not combine chemically with acids but form adsorption compounds. Chapman, Greenberg and Schmidt [1927] find that in solutions more acid than p_H 2·5 certain dyes combine with proteins in stoicheiometric proportions giving protein-dye precipitates. The valency of the proteins in these compounds approximates closely to the maximum acid-combining power of the proteins calculated from their diamino-acid content. Gortner's [1927] reply is that these results "offer very striking substantiation of the conclusions reached by Hoffmann and Gortner." Gortner assumes that in very acid solution there are present protein cations with surface electrical charges and the number of these charges is equal to the chemical valency of the protein, and the same with the dye. These charges are neutralised and a protein-dye colloid is precipitated. It seems probable, however, that such neutralisation of ionic charges may well be chemical combination, and is in no wise different from the precipitation of barium sulphate by the neutralisation of the ionic charges of barium and sulphate ions and formation of an insoluble chemical compound. The mere fact of the protein cation being larger than the barium cation does not change a phenomenon from chemical combination to physical adsorption.

SUMMARY.

Phthalein and fluorescein dyes which change colour on the acid side of the isoelectric point of proteins combine with proteins in acid solution giving coloured oxonium salts. The colour of such dyes is not discharged or altered as usual on acidification and hence they cannot be used as indicators in solutions containing proteins. The "protein error" of indicators, acid-fast staining of tissue sections and acid-fast dyeing of fabrics are explained.

When any phthalein or fluorescein dye is mixed with a protein solution and acid is added, a precipitate forms at the isoelectric point of the protein and dissolves at about p_H 2·5. Salt formation is indicated, and it is considered unnecessary to postulate physical adsorption to explain any of the phenomena described.

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CLXXIII. THE SURFACE TENSION OF PROTEIN SOLUTIONS. PART III.

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The surface tension of protein solutions has been given a definite meaning by the work of du Noüy [1926] who has shown strikingly the adsorption of the protein molecule at the surface, forming there an organised unimolecular layer similar in every respect to the orientated unimolecular films of fatty acids and other long chain organic compounds of Langmuir and Adam. Du Noüy's calculations of the dimensions of the egg-albumin molecule from surface tension measurements, which give a value for molecular weight in close agreement with those of other observers, are further evidence that the colloidal behaviour of protein solutions is due to the size of the single molecule, although agglomeration into micellae may occur under certain conditions. Thus, for a given concentration, volume of solution and area of surface, any alteration, chemical or physical, in the molecule will affect the surface tension by the alteration of the surface layer.

In previous communications [1925, 1926] the Donnan equilibrium was considered to affect the surface tension of a protein solution according to the Lippmann effect of the charge on the particle, which Loeb [1922, 1] has proved to be due to the potential difference between the particle and the solution. In view of du Noüy's researches establishing the existence of an organised surface layer in protein solutions, it can be seen that the greater the charge on the molecules of protein, the greater the repulsive forces between adjacent molecules, hence the lower the cohesive forces and the tensile strength of the surface layer.

The earlier work on surface tension of gelatin solutions showed, however, that this is not the only factor affecting the surface tension of protein solutions, the solubility, hydration and denaturation of the protein molecule also being important influences independent of a membrane equilibrium which will alter the manner of the packing of the surface layer.

In the present communication the effects on the surface tension of an alteration in the protein molecule due to solubility, change of reaction, presence of neutral salts, and denaturation were studied for caseinogen and egg-albumin in order to extend the previous work of St Johnston and Peard [1925, 1926].

EXPERIMENTAL.

The method used both in the present and previous work for the determination of the surface tension was the maximum bubble pressure method of Sugden [1922, 1924]. This method, together with hanging-drop methods of which it is the inverse, is open to the criticism that it is comparative only. Du Noüy [1926] has also shown the importance of the time factor in the determination of the surface tension of colloidal solutions due to the slow rate of adsorption of the particles or molecules at the surface; he states, "It is thus essential when giving the surface tension of a colloidal solution to state the time which has elapsed since the solution was violently agitated, and, unless the method is a true static one, for results to be even comparable amongst themselves, everything being otherwise equal, the time required for the formation of a drop or bubble must be about the same for a large number of drops." Wolf [1923] has shown for bacteriological media that the surface tension as determined by a modification of du Noüy's static ring method is about 10 dynes lower than that determined by the drop method with a fast rate of dropping, whereas when the time of dropping was much prolonged agreement between the two methods was close.

The bubble method employed in this investigation was carried out under standard conditions with an approximately constant bubble rate, and the mean of several readings taken for each determination. Since the solutions employed were relatively concentrated compared to those of du Noüy, equilibrium at the surface would be attained much more rapidly owing to the more rapid diffusion towards the surface layer. Again, the pressure difference between two jets is observed; hence the effect of small variations in the bubble rate is partly compensated.

A further objection to Sugden's method when applied to colloidal solutions has been made by King [1925] on account of the frothing due to the bursting of the bubbles. Peard and St Johnston [1925], however, have shown for brewery worts that the amount of protein removed in this manner is negligible in the course of a determination of the surface tension, whilst frothing at the surface of the solution may be very much reduced or entirely prevented by the presence of a trace of toluene there without affecting the surface tension at the fresh bubble surface formed within the bulk of the liquid.

The determinations were carried out at 25° and the solutions were prepared 24 hours beforehand and kept at 25° to ensure chemical equilibrium being attained, a drop of toluene being added to ensure sterility and to prevent frothing. The bubble rate on the small jet was timed for each reading and was at a rate of one bubble in 1–3 seconds, but no appreciable constant difference was found experimentally between the pressure-difference readings for one bubble in one second and one in five seconds. The mean of five or six readings at various bubble rates within the above limits was taken as the value of the surface tension required. All the instruments used had jets of approximately the same size, were calibrated with pure benzene and checked with water; the pressure difference readings for water were from 7 to 11 cm. on the alcohol gauge for various instruments, and all gave values for the surface tension of water within 0·5 %. They also agreed with each other to that extent when checked with the same protein solution. All the containers used were cleaned with chromic acid and washed and dried. Corks or rubber bungs were covered with tinfoil. The surface tension instruments were cleaned with nitric acid and well washed between each determination. In the preparation of solutions only boiled distilled water kept sterile and CO_2-free was used, and for alkaline solutions a small soda-lime tube was attached to the bubbler of the surface tension instrument to prevent any access of CO_2 from the air. 5 cc. of solution were required for a determination.

The p_H determinations below p_H 7·5 were made at 20° with the quinhydrone electrode, using a gold electrode according to the technique of Corran and Lewis [1924] and 0·03 g. ground quinhydrone crystals per 20 cc. of solution. Above p_H 7·5 the determinations were made with the hydrogen electrode.

The caseinogen used was a commercial pure preparation which was fat-free and contained 9·58 % moisture, 0·11 % ash and 14 % N. It was therefore deemed suitable for use without further purification, being ash-free except for that organically combined, and, taking 6·47 as the protein factor, it contained 90·6 % caseinogen. In all experiments 0·56 g. of this caseinogen was dissolved in 50 cc. of solution containing the requisite quantity of $N/10$ acid or alkali to give a 1 % solution.

The crystallised egg-albumin was prepared from new-laid eggs according to the method of Sørensen [1915], recrystallised twice and dialysed for 3 weeks at 5°. Owing to the somewhat prolonged dialysis and slight leaks, the resulting albumin solutions were only about 0·6 %, so that when diluted with the requisite amount of $N/10$ acid or alkali they were reduced to 0·3 %. This was, however, sufficient to give a marked lowering of surface tension. Solution A contained 0·92 mg. protein-N and 0·016 mg. ammonium sulphate-N per cc. and had p_H 5·6; it was faintly turbid. Solution B contained 0·96 mg. protein-N and 0·036 mg. ammonium sulphate-N per cc. and had p_H 6·76; it was quite clear.

The effect of time on the surface tension of caseinogen solutions.

Owing to the low solubility of caseinogen, a considerable time was required for solution to take place, even when the conditions were favourable for complete solution. It was therefore necessary to determine the effect of the age of the solution on the surface tension, in order that future determinations of surface tension should be made when equilibrium had been attained. Caseinogen will only give completely dissolved 1 % solutions with hydrochloric and phosphoric acids between p_H 3·0 and 1·6, and with sodium hydroxide of p_H greater than 7·5. The effect of time on the surface tension of 1 % caseinogen solutions in HCl at p_H 2·46 is shown in Table I. Solution in acids occurs with swelling and there is a rapid fall of surface tension in the first few minutes followed by a slower fall during about 24 hours, after which there is little change. The solutions were opalescent at first and did not clear for about 12 hours. Similarly with a partial solution in sodium hydroxide, p_H 6·8, equilibrium was not reached until nearly 24 hours had elapsed, although in alkali no swelling occurs.

Henceforth all determinations of surface tension of 1 % caseinogen solutions or suspensions were made 24 hours after preparation.

Table I. *Effect of age of solution of caseinogen on the surface tension.*

1 % caseinogen in *N*/100 HCl at 25° (2 experiments)

Time	Surface tension (*a*) Dynes per cm.	Surface tension (*b*) Dynes per cm.
2 mins.	71·5	64·9
4 ,,	70·8	62·6
6 ,,	70·2	61·5
10 ,,	69·5	60·5
15 ,,	63·1	60·5
20 ,,	61·9	—
50 ,,	61·5	—
60 ,,	61·0	59·7
2 hours	60·5	59·2
6 ,,	59·5	—
18 ,,	59·0	58·5
24 ,,	58·7	58·6
43 ,,	—	58·5

1 % caseinogen in *N*/250 NaOH at 25°

Time	Surface tension Dynes per cm.
2 mins.	70·8
5 ,,	60·1
8 ,,	59·6
12 ,,	59·0
15 ,,	58·6
30 ,,	58·1
45 ,,	58·0
60 ,,	58·0
2 hours	58·0
19 ,,	57·0
26 ,,	56·9
43 ,,	56·3

The effect of p_H on the surface tension of caseinogen solutions.

The titration curves of caseinogen in presence of HCl and H_3PO_4 were identical with those of Loeb [1922, 1], but the curve obtained for caseinogen in presence of *N*/10 NaOH differed from Loeb's, being always less alkaline for a given amount of NaOH added, particularly on the part of the curve where the change of p_H with increased addition of alkali is most rapid, *i.e.* between p_H 6 and 10. A regular curve was however obtained, and values of p_H determined with the hydrogen electrode agreed well with those determined by the quinhydrone electrode up to the limit where the use of the latter is possible. A clear 1 % solution of caseinogen in NaOH was not obtained below p_H 7·5. The rapid hydrolysis of caseinogen by alkali when the p_H is greater than 10 where a change in direction of the titration curve is apparent has been pointed out by Zoller [1921]. It was noted that solution took place very rapidly in liquids alkaline to p_H 10 and that all power of "head" retention was lost, foam formed on shaking disappearing immediately. The results of the variation of the surface tension with p_H are shown in Fig. 1. It will be seen that the general form of the curve is similar to that obtained by St Johnston and Peard [1925, 1926] for gelatin, the maximum surface tension being at the iso-electric point, p_H 4·62, as expected from Donnan's theory, though for caseinogen the value did not rise to that of pure water as in the case of gelatin, being 67·8 dynes per cm. Robertson [1918] and Cohn and Hendry [1923] have, however, shown that caseinogen is slightly soluble in water when pure, even at the isoelectric point. The fall of surface tension is very rapid on either side of the isoelectric point and there is a minimum of surface tension on the acid side at p_H 3·8–3·9 followed by a well-marked secondary maximum at p_H 3·4, such as was obtained for gelatin at p_H 2·0–2·2. This maximum at p_H 3·4 is just before the caseinogen becomes completely soluble (about p_H 3·0). In presence of H_3PO_4 the surface tension proceeds parallel to that in presence of HCl but is uniformly a dyne lower. The maximal values of the osmotic pressure and viscosity of caseinogen in presence of HCl are at p_H 3·0, and Zoller [1921] states that "acid hydrolysis of caseinogen must of necessity commence somewhere in the neighbourhood of this drop or flattening of the curve." St Johnston and Peard [1925], however, were unable to find in the case of gelatin solutions acid to p_H 2·2 any evidence of hydrolysis as evinced by formaldehyde titration commencing suddenly below p_H 2·2. This point is dealt with later. The minimum value p_H 8·5 agrees fairly well with Zoller's value of p_H 9·0 for the maximum of viscosity of caseinogen in alkaline solution. Owing to the limited solubility above p_H 3·0 it would not be expected that the maxima of viscosity and osmotic pressure of caseinogen solutions would agree with the minimum surface tension, since the degree of solubility greatly influences the properties, in fact Loeb [1922, 1] states that the maximum for the influence of caseinogen hydrochloride on viscosity lies at p_H equal to or greater than 3·0. The surface tension curve seems to indicate that the Donnan equilibrium gives a maximum potential difference at a higher p_H than 3·0, since the minimum surface tension is at p_H 3·8–3·9. If solubility were the only factor affecting the surface tension no minimum would be observed.

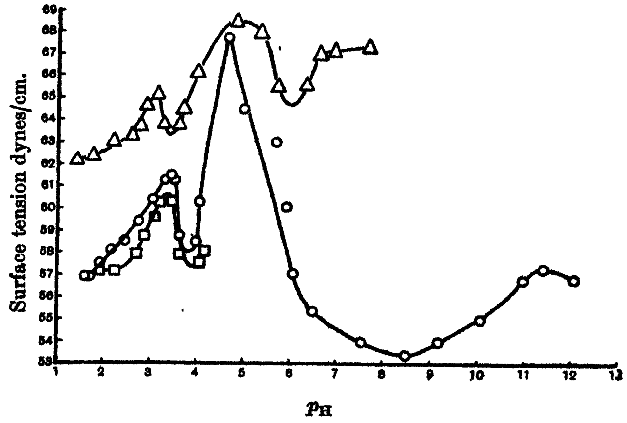

Fig. 1. Effect of p_H on the surface tension of caseinogen and egg-albumin solutions.

○ 1 % caseinogen in presence of HCl and NaOH
□ " " " H_3PO_4
△ 0·3 % egg-albumin " HCl and NaOH

On the alkaline side the fall of surface tension was much greater, the minimal value being at p_H 8·5, followed by a rise in surface tension with increasing alkalinity with a possible secondary maximum at about p_H 11·5.

The effect of p_H on the surface tension of egg-albumin solutions.

A similar series of determinations of surface tension at various hydrogen ion concentrations within the range of the quinhydrone electrode was carried out with crystallised egg-albumin solutions. The stock solution of albumin purified as described above was mixed with the requisite quantity of HCl or NaOH to give a final concentration of 0·3 % albumin. The solutions were kept for 24 hours at 25° before determining the surface tension at 25°. The results are also shown in Fig. 1, where it will be seen that the maximum surface tension of egg-albumin solutions is also at the isoelectric point, p_H 4·8. Like caseinogen, it does not attain the value for pure water as flocculated isoelectric gelatin does, which is as expected from the solubility of isoelectric albumin. Loeb [1922, 2; 1923] has also shown that neutral salts even in very low concentrations may impart a surface potential to isoelectric denatured albumin, and experiments on the effect of neutral salts on protein solutions to be described later also show that in low concentrations they decrease the surface tension; hence the presence of ammonium sulphate even in concentration of $M/3000$ will tend to lower the surface tension of isoelectric albumin solution, accounting for the value of 68·5 dynes/cm. instead of the theoretical value 72·2 dynes/cm. of pure water.

A sharp secondary maximum of surface tension on the acid side of the isoelectric point occurs at p_H 3·0. The minimum between these maxima is at p_H 3·4–3·5; thus the maximum lowering of surface tension agrees well with Loeb's values of p_H 3·4 for the maximum osmotic pressure and p_H 3·6 for the maximum membrane potential.

So far as the alkaline side has been investigated there is a minimum surface tension at p_H 6·0. Loeb found the potential difference of albumin to be still increasing at p_H 5·8 from the minimum value at the isoelectric point.

As discussed in an earlier communication [St Johnston and Peard, 1925], the surface tension of a protein solution should be a maximum when the potential difference is a minimum and *vice versa*, thus, as in the cases of gelatin and caseinogen, the fall of surface tension of egg-albumin solutions is explicable up to a point by Donnan's membrane equilibrium theory.

The probable cause of the further fall in surface tension in more acid solutions, observed in all the proteins investigated, giving rise to the second maximum of surface tension, which is not accountable by the Donnan equilibrium will be dealt with in a succeeding section.

The effect of neutral salts on the surface tension of caseinogen solutions.

Since caseinogen is only completely soluble in HCl between about p_H 1·6 and 3·0, a solution of 1 % caseinogen in $N/100$ HCl of original p_H 2·46 was used to study the effect of neutral salts on the surface tension in acid solution. Since on the acid side of the isoelectric point the influence of the anion is greatest, all the salts investigated had the same cation, sodium, to eliminate any possible effect due to the cation. The salt to be added was weighed out and dissolved in the solution at the same time as the caseinogen, keeping the solution for 24 hours at 25° as usual. The effect of $NaNO_3$, NaCl and Na_2SO_4 and also of a non-electrolyte, glucose, is shown in Fig. 2. All the salts, in spite of a difference in valency of the anion, produce the same lowering of surface tension below that of the original caseinogen solution up to a concentration of $M/256$, and in greater concentrations produce a rise in surface tension, which is greater for Na_2SO_4 than for $NaNO_3$ and NaCl. The effect of glucose on the surface tension was practically negligible.

In experiments on the influence of neutral salts on protein solutions, the effect of the neutral salts on the p_H must always be considered. In all cases the p_H as determined electrically was increased slightly by the addition of a salt, in proportion to the concentration added, as was observed by Chick and Martin [1911]. NaCl and $NaNO_3$ at the same concentration raised the p_H to approximately the same extent, whilst Na_2SO_4 caused about double the rise due to NaCl or $NaNO_3$ at the same concentration. The rises of p_H were, however, not enough to account for the changes in surface tension observed. At p_H 2·46 it will be seen from Fig. 1 that an increase in p_H should raise the surface tension; up to $M/256$ for all three salts a fall of 2 dynes was observed, whereas the p_H changed from 2·46 to 2·52 for NaCl and $NaNO_3$ and to 2·66 for Na_2SO_4, which should have raised the tension by 0·2 and 0·6 dyne respectively.

At $M/32$ the surface tension in presence of NaCl and $NaNO_3$ had risen again so that it was only 1 dyne below the original value, whilst the p_H had increased to 2·64, a rise of p_H 0·18, which should increase the surface tension by 0·5 dyne. Na_2SO_4 at $M/32$ raised the surface tension 3 dynes above the original value, with an increase of p_H 0·36, which alone would only increase the surface tension by 1 dyne. Thus the changes in surface tension observed in presence of neutral salts are far greater than can be accounted for by the changes in p_H produced.

If the surface tension of protein solutions depended only on the Donnan effect at the p_H of the above experiment, addition of neutral salts even in lower concentrations than $M/1024$ should depress the potential difference, as shown by Loeb [1922, 1], and therefore raise the surface tension, and the effect should be in proportion to the valency of the anion added. Evidently this is not what takes place in concentrations below $M/256$ since the sulphate ion has exactly the same effect as the chloride and nitrate ions in lowering the surface tension. A possible cause of the fall of surface tension in presence of neutral salts below a concentration of $M/256$ is that denaturation of the protein is increased by their presence. Mastin and Schryver [1926] have shown that denaturation of egg-albumin occurs on standing in presence of acid and neutral salt, and in a succeeding section it will be shown that denaturation produces a lowering of surface tension and that denaturation is occurring during the 24 hours at 25° in solutions of caseinogen at p_H 2·46 or of albumin at p_H 2·6. This may be increased by the presence of salts thus lowering the surface tension, though in greater concentration than $M/256$ the Donnan effect and the lyotropic effect of removal of associated water predominate and raise the surface tension, so that when the salt concentration is sufficiently great the protein is salted out completely and the surface tension becomes that of the salt solution.

The effect of a neutral salt on the surface tension of albumin solutions.

The effect of Na_2SO_4 upon 0·3 % albumin solution in presence of HCl was followed in the same manner as for caseinogen, and found to produce the same results. The p_H of the solutions was originally 2·6, which is at a similar point to a caseinogen solution of p_H 2·46 in respect to the surface tension-p_H curves of Fig. 1. The p_H rose with the addition of Na_2SO_4 up to a value of p_H 3·0 when in presence of M Na_2SO_4.

No sign of precipitation occurred up to a concentration of $M/16$ Na_2SO_4, where there was slight turbidity. With $M/4$ Na_2SO_4 there was much precipitation and with M Na_2SO_4 (nearly saturated solution) the albumin was salted out almost entirely.

The effect on the surface tension is also shown in Fig. 2, where it will be seen that there is a similar fall of surface tension to that of caseinogen solutions to a minimum at $M/256$, followed by a rise. After $M/16$, however, the rise is much increased, when the albumin is almost entirely salted out and the surface tension is nearly that of water. Evidently in the stronger solutions the lyotropic effect of the salt in withdrawing associated water from the protein is acting as well as the effect due to the salt on the Donnan equilibrium.

The denaturation of egg-albumin solutions in presence of acids.

In a previous communication [St Johnston and Peard, 1926] it was suggested that the fall in surface tension of gelatin solutions acid to the maximum of surface tension at p_H 2·0–2·2 might be due to hydrolysis or denaturation of the protein. No evidence of immediate hydrolysis as shown by formaldehyde titration was found however, though on standing for some months the most acid solutions had increased in formaldehyde titre and the surface tension had fallen further. Harris [1923], also from formaldehyde titrations on fresh and denatured egg-albumin, came to the conclusion that no detectable change occurred on denaturation, although there is a change in the sulphur linkages, and Lewis [1927] found no detectable change in titratable acidic or basic groups of haemoglobin on denaturation. Young [1922] found that light-denatured albumin had a lower surface tension than before exposure.

Since caseinogen and egg-albumin behave similarly to gelatin in respect to the effect of p_H on the surface tension, experiments were undertaken with egg-albumin to ascertain if the fall in surface tension in the more acid solutions, which is not in accordance with the membrane equilibrium theory, could be ascribed definitely to denaturation. Whatever the nature of the change in the protein on denaturation, it is possible that it might lower the surface tension.

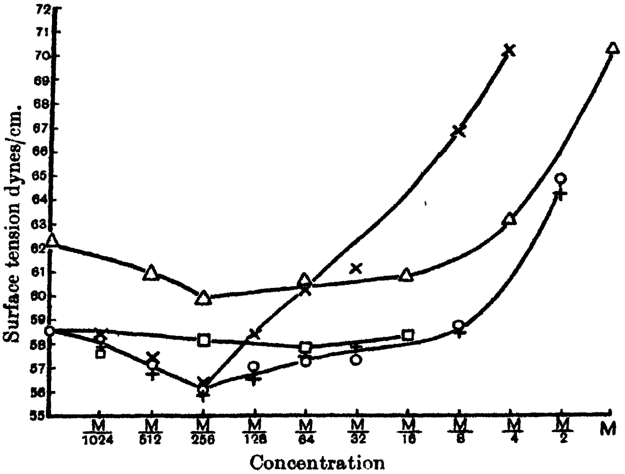

Fig. 2. Effect of neutral salts on the surface tension of caseinogen and egg-albumin solutions.

○ 1 % caseinogen in presence of $NaNO_3$ and HCl originally p_H 2·46
+ ,, ,, ,, NaCl ,, ,, p_H 2·46
× ,, ,, ,, Na_2SO_4 ,, ,, p_H 2·46
□ ,, ,, ,, glucose ,, ,, p_H 2·46
△ 0·3 % egg-albumin ,, Na_2SO_4 ,, ,, p_H 2·60

Egg-albumin, free from salts, is not coagulated by heating, whilst denaturation is greatly accelerated by heating; thus a dialysed albumin solution is very suitable for studying the effect of denaturation on surface tension, since if coagulation occurred there would inevitably be a rise of surface tension to that of pure water on complete coagulation. The solutions employed were of such a salt concentration that only those between the isoelectric point and neutrality were coagulable. As will be seen from Fig. 1, the surface tension of albumin solutions varies with the p_H in a manner explicable by Donnan's membrane equilibrium theory between p_H 3·0 and neutrality, but below p_H 3·0 the surface tension again falls sharply with increasing acidity. If this further fall is due to denaturation occurring while standing for 24 hours at 25° in presence of acid giving a p_H less than 3·0, the effect should become more marked on accelerating the denaturation by heating.

Sørensen [1925] has shown that coagulation proceeds most rapidly near the isoelectric point, whilst the preceding process of denaturation occurs more rapidly the greater the hydrogen ion concentration. Somewhat similar experiments were therefore carried out and the surface tensions observed. A recently dialysed, clear solution of albumin (B) was used, and 0·3 % solutions of various p_H were kept at 25° for 24 hours. The salt content was about $M/1500$ $(NH_4)_2SO_4$ which was insufficient to cause coagulation of the denatured protein on heating except in the region of the isoelectric point. The surface tension was determined at 25°, then the solution, still in the surface tension bubbler, was put in a water-bath at 60° for exactly 15 minutes, quickly cooled under the tap and the surface tension redetermined at 25°.

Chick and Martin [1911, 1912] have shown a fixation of acid on denaturation of egg-albumin. It was therefore necessary to determine the changes in p_H occurring under the conditions of the experiment. Another preparation of dialysed albumin was used for this purpose and the p_H of the 0·3 % solutions determined after they had stood 24 hours at 25° and again after heating for 15 minutes at 60°. It will be seen from Table II that the only appreciable changes in p_H occur between p_H 3·8 and p_H 2·5, which show a maximum increase of p_H 0·15; this would have some effect on the surface tension, but it will be seen from Table III that over the range of p_H 3·0–4·0 there is practically no change of surface tension. From Fig. 1 it will be seen that a rise of p_H between 2·5 and 3·0 would tend to raise the surface tension, whereas from Table III at p_H 2·85 it falls. Between p_H 3·0 and 3·5 increase of p_H would lower the surface tension and between 3·5 and the isoelectric point raise it, only at p_H 4·0 does this begin to occur, when coagulation is also beginning. Thus the change of p_H on denaturation by no means accounts for the changes of surface tension observed, and where the maximum fall of surface tension is observed no change of p_H occurs. That no change occurs on further heating the most acid solutions seems to be due probably to the fact that a certain amount of denaturation and consequent fixation of acid has taken place while standing for 24 hours at 25°.

Table II.

Original p_H	p_H after heating 15 mins. at 60°	Change of p_H
6·63	6·64	+·01
3·78	3·93	+·15
3·33	3·47	+·14
2·78	2·91	+·13
2·54	2·62	+·08
2·38	2·39	+·01
2·24	2·25	+·01
1·87	1·87	0
1·70	1·71	+·01

The surface tension results are recorded in Table III.

Thus in solutions alkaline to and including the isoelectric point a heavy coagulation accompanied by a rise in surface tension occurred, and at the isoelectric point the surface tension rose to that of water.

Table III.

p_H	Surface tension		Change of surface tension (dynes per cm.)	Appearance after heating
	Before heating (dynes per cm.)	After 15 mins. at 60° (dynes per cm.)		
6·76	67·9	70·2	+2·3	Coagulation
6·28	65·5	68·6	+3·1	,,
5·32	68·0	71·3	+3·3	,,
4·77	68·5	72·1	+3·6	,,
4·00	65·9	68·0	+2·1	Slight turbidity
3·66	64·0	64·0	0	Clear
3·22	63·8	63·9	+0·1	,,
3·10	64·7	64·6	−0·1	,,
2·85	63·7	63·1	−0·6	,,
2·03	63·3	62·5	−0·8	,,
1·79	63·3	62·2	−1·1	,,

The solution of p_H 4·0 showed a marked rise in surface tension with only a very slight turbidity, whilst solutions between p_H 3·0 and 4·0 gave no change of surface tension or of appearance.

From the point at which the solutions became acid to the maximum of surface tension at p_H 3·0 a marked fall in surface tension occurred on heating, the fall increasing with increasing hydrogen ion concentration.

The experiment was carried a stage further by heating the solutions to 60° for 15 minutes, then bringing to the isoelectric point by the addition of a buffer solution and again heating. In this case an albumin solution (A) which had been prepared about 6 weeks previously was used. This solution, again of 0·6 % concentration but with only about $M/3000$ $(NH_4)_2SO_4$ and saturated with toluene, had been kept at about 4° in the dark and had become opalescent with a slight deposit, agreeing with the observation of Sørensen [1926] who found a similar change under the same conditions, namely, a slight deposit of denatured albumin, whilst the filtered solution could be recrystallised, giving material with normal properties, except that it contained less phosphorus than fresh albumin. The solution was filtered and solutions of various p_H prepared and kept at 25° for 24 hours as before. The surface tensions were found to be lower than the values previously determined for the same p_H; thus some change had occurred, but this was immaterial to the experiment.

The results are given in Table IV.

Table IV.

(*a*) Experiments as in Table III.

p_H	Surface tension		Change of surface tension (dynes per cm.)	Appearance after heating
	Before heating (dynes per cm.)	After 15 mins. at 60° (dynes per cm.)		
4·9	64·5	69·4	+4·9	Coagulation
4·0	62·2	65·2	+3·0	Slight turbidity
2·6	62·1	61·0	−1·1	Clear
1·43	61·4	60·1	−1·3	,,

(*b*) Equal volume 0·2 *N* acetate buffer, p_H 4·87, added to above heated solutions.

		Surface tension after			
Original p_H	p_H after buffer added	Buffer added (dynes per cm.)	Heating 15 mins. at 60° + buffer (dynes per cm.)	Change of surface tension (dynes, per cm.)	Appearance after heating
4·9	4·81	69·5	69·8	+0·3	Coagulation still present
4·00	4·88	67·5	67·6	+0·1	Slightly increased turbidity
2·60	4·81	64·0	67·8	+3·8	Ditto.
1·43	4·56	64·8	66·4	+1·6	Ditto.

(*c*) Control experiment: 0·2 *N* acetate buffer, p_H 4·87, added to solutions as above not previously heated at 60°.

		Surface tension after			
Original p_H	p_H after buffer added	Buffer added (dynes per cm.)	Heating 15 mins. at 60° (dynes per cm.)	Change of surface tension (dynes per cm.)	Appearance after heating
4·9	4·81	66·5	66·5	0	No coagulation
4·00	4·88	66·4	66·9	+0·5	,,
2·60	4·81	64·8	65·4	+0·6	,,
1·43	4·56	65·2	65·6	+0·4	,,

The effect of heating for 15 minutes at 60° was the same as before, and on the addition of an equal volume of an 0·2 *N* acetate buffer, p_H 4·9, and again heating for 15 minutes at 60°, rises of surface tension were now observed in those solutions which had previously been acid to p_H 3·0 but not in the others. There was no coagulation after adding the buffer, though for surface tension determinations this was an advantage. Control experiments were made by adding the buffer to equal volumes of solutions which had not been heated at 60°; these were thus only heated at that temperature in the neighbourhood of the isoelectric point. It was observed that the presence of the buffer prevented the coagulation of the nearly isoelectric albumin, which coagulated readily when heated alone. Lepeschkin [1922] found that some salts have an accelerating and others a retarding effect on the coagulation-velocity of denatured albumin, whilst Lewis [1926] found the denaturation-velocity accelerated or retarded according to the salt and its concentration. Apparently with the relative concentrations present here, namely 0·15 % albumin and 0·1 *N* acetate, there is a retardation sufficient to prevent coagulation during 15 minutes at 60°.

The control experiment, Table IV (*c*), shows that a slight amount of denaturation during the 24 hours at 25° has occurred in all but the isoelectric solution, as, although no visible coagulation occurred, the surface tension rose slightly on heating in presence of the buffer. The solution originally most acid was not brought quite to the isoelectric point by the addition of the buffer, hence the rise of surface tension was not as great as for the other two acid solutions, both in the control experiment and in experiment IV (*b*), although in experiment IV (*a*) it showed the greatest fall of surface tension after heating without buffer. Since denaturation is increased by heating, the falls of surface tension in acid solutions after heating recorded in Tables III and IV (*a*) clearly indicate that denaturation is accompanied by a fall of surface tension. Coagulation is accompanied by a rise in surface tension as would be anticipated and is well shown by those solutions in Table III giving visible coagulation; thus in those solutions heated with buffer near the isoelectric point the rises of surface tension are due to coagulation of the previously denatured protein although it is insufficient to be visible. With the above-mentioned exception of the most acid solution, p_H 1·43, the greatest rises of surface tension on heating with buffer occur in those acid solutions which showed the greatest falls on previous heating without buffer. This further proves the falls in surface tension to be due to denaturation, since Sørensen [1925] has shown that the greatest amount of coagulation when brought to the isoelectric point occurs in those solutions which had previously been most denatured, namely, those with the greatest hydrogen ion concentrations originally.

The experiments bring out most clearly the marked increase in denaturation after p_H 3·0 which is the point of inflexion of the surface tension-p_H curve of albumin, hence the previous assumption that this was due to denaturation seems to be correct. Recent work by Mastin and Schryver [1926] has shown that denaturation of egg-albumin is brought about by long standing in the presence of acid and salt, and Chick and Martin [1911, 1912] and Lewis [1926] have proved the denaturation velocity to increase rapidly with the H or OH ion concentration from a minimum at neutrality. Hence there will be a certain amount of denaturation occurring in an egg-albumin solution during 24 hours at 25°, increasing with the acidity, and the above experiments show that a sudden great increase occurs after p_H 3·0. Since gelatin and caseinogen have similar surface tension-p_H curves to egg-albumin, with acid maxima at p_H 2·0 and p_H 3·4 respectively, it is reasonable to assume for these proteins also that denaturation is the cause of the fall of surface tension in solutions acid to these points.

Thus denaturation also accounts for the fall in surface tension of gelatin solutions on keeping over a long period, as reported previously [St Johnston and Peard, 1926], and for the low values obtained with albumin solution A given in Table IV compared with the values in Fig. 1 obtained some weeks previously.

CONCLUSION.

Although du Noüy has shown the surface tension phenomena of protein solutions to be due to the single molecules of proteins, the surface tension may be brought into line with other properties of colloidal behaviour as being dependent upon the Donnan equilibrium except where denaturation occurs or where adsorption or lyotropic effects of neutral salts predominate over the membrane equilibrium effect. As was remarked by Michaelis [1926], and would be expected since the charge on the particle is a minimum, the maximum surface tension is at the isoelectric point for the three proteins investigated, gelatin, caseinogen and egg-albumin, whilst the hydrogen ion concentrations of the maximum lowerings of surface tension on either side of the isoelectric point agree fairly well with those of the maxima of osmotic pressure, viscosity and potential difference found by Loeb [1922, 1] for the same proteins.

The published results with other proteins are somewhat at variance, particularly in respect to the surface tension at the isoelectric point. Buglia [1908], Bottazzi [1912, 1913], Bottazzi and d'Agostino [1912, 1913] found the surface tension of proteins, in particular those of the body fluids, to vary with the acidity or alkalinity much as described above, though of course the p_H was not considered. Whereas in some cases suspensions of a serum-protein in water did not appreciably lower the surface tension, with other serum-proteins a minimum surface tension at the isoelectric point was apparently observed, and Bottazzi's inference that the undissociated protein produces the greatest lowering of surface tension has been generally quoted. Although Quagliariello [1921] found the same for haemoglobin, he considers it anomalous. Bottazzi's view was apparently confirmed for gelatin by de Caro [1925] but this was shown not to be so in a previous communication [St Johnston and Peard, 1926] and the maximum surface tensions of caseinogen and egg-albumin at their isoelectric points are a further confirmation that this is not the case.

The sensitiveness of surface tension determinations of protein solutions to slight differences of p_H, to denaturation and to small amounts of neutral salt seems a likely explanation of the variable results with other proteins, particularly as in most cases no account was taken of p_H.

Hence it appears that surface tension lowering is a general property of the proteins which varies with the hydrogen ion concentration, as do other properties dependent upon the Donnan equilibrium, but the influence of denaturation appears in more acid solution and in presence of neutral salts.

SUMMARY.

1. The surface tension of caseinogen and egg-albumin solutions varies with the p_H similarly to that of gelatin solutions, being maximum at the isoelectric point and minimum at points corresponding to the maxima of other properties, whilst a secondary maximum occurs in more acid solutions.

2. Neutral salts in low concentration lower the surface tension of caseinogen and egg-albumin in acid solution, but raise it in a greater concentration.

3. Denaturation of egg-albumin lowers the surface tension, which accounts for the fall of surface tension in solutions acid to the secondary maximum in the surface tension-p_H curve, for the initial fall in presence of low concentrations of neutral salts, and for the fall in surface tension on long standing.

4. The surface tension of protein solutions appears to be a general property which can be brought into line with the other properties of proteins as being governed by the Donnan equilibrium when due allowance is made for denaturation and the lyotropic effect of neutral salts.

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CLXXIV. A NEW COLORIMETER BASED ON THE LOVIBOND COLOUR SYSTEM, AND ITS APPLICATION TO THE TESTING OF COD-LIVER OIL, AND OTHER PURPOSES.

BY OTTO ROSENHEIM,

WITH AN ADDENDUM BY EDGAR SCHUSTER.

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THE need for the construction of an apparatus of the type described in this communication[1] arose during the course of work on the colorimetric estimation, by means of the arsenic—or antimony—trichloride reaction, of the chromogen contained in cod-liver oil and other fats, which is assumed to be identical with vitamin A [Rosenheim and Drummond, 1925; Carr and Price, 1926]. The difficulties which had to be overcome before this test could be applied quantitatively were twofold and were due (1) to the characteristic instability and transient nature of the brilliant blue colour developed in the test, and (2) to the variable tints of blue (*i.e.* greenish or reddish) produced and determined by the uncontrollable and variable admixture of yellow or red pigments in the oils to be tested. The formation of these adventitious colours excluded the employment of any one fixed standard for comparison, as well as the use of a colorimeter of the usual Duboscq type. Lovibond's Tintometer, on the other hand, enables a perfect matching of any mixture of colours to be made, but the apparatus in its present form is hardly suitable for the extremely rapid work essential in the above test. Considerable experience is required in the expeditious selection of the suitable standard glasses, which have to be inserted into the apparatus singly and exchanged by hand if unsuitable. This drawback has been overcome in the model illustrated (see Fig. 1) by arranging the standard glasses in frames containing ten each of the red, yellow and blue units, in such a way that they can be rapidly moved horizontally behind a window and below the cell (or test-tube) containing the coloured solution to be matched. A mounting of the standard glasses on discs was considered in the first instance. The horizontal arrangement, in preference to a vertical one, was chosen for technical reasons in discussion with Dr W. V. Thorpe, to whom I am also indebted for the construction of a small working model. The apparatus now described, which is entirely different in general arrangement and mechanical details from Dr Thorpe's model, was designed and constructed by Dr E. Schuster, who has kindly furnished the description of it given in this paper.

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[1] The Tintometer, Ltd., Salisbury, have undertaken the manufacture of the apparatus, all rights in it having been put freely at their disposal.

The apparatus has proved itself not only eminently suitable for the purpose for which it was designed, but possesses certain advantages, which seem to justify a recommendation for its use in colorimetric work generally and in biochemistry in particular. Estimations made with it are free from the error inherent in those obtained by the use of Duboscq's colorimeter, or any of its modifications, in which two coloured solutions of different concentration are compared in layers of different thicknesses. This error, which may in extreme cases amount to as much as 25 %, is discussed fully by Stanford [1913, 1923]. Further, a well-known difficulty in biochemical colorimetric work, which cannot be overcome in the usual type of colorimeter, is due to the difference in tint between the colours given by the "unknown" and by the standard prepared from a pure substance respectively. The formation of adventitious

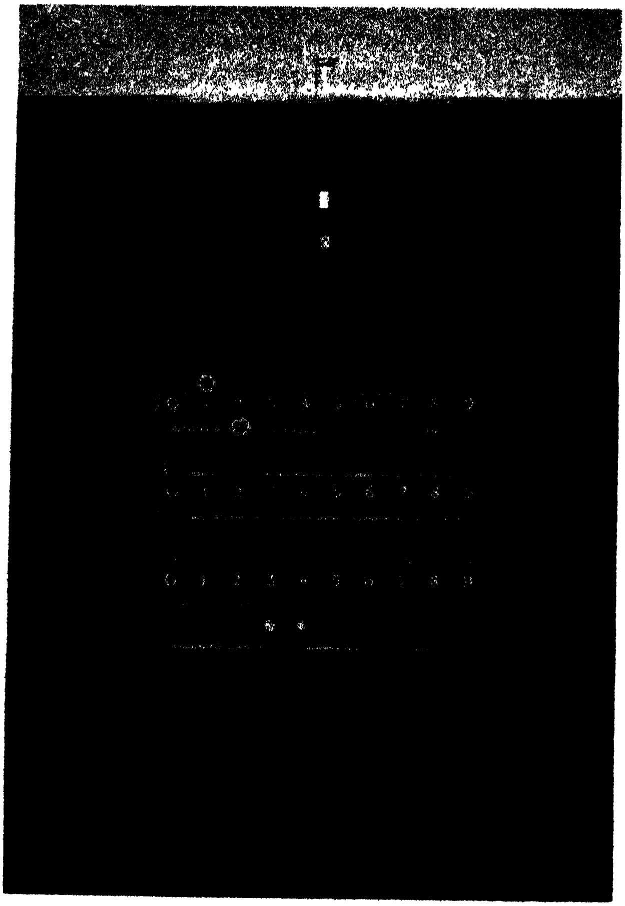

Fig. 1.

colours due to constituents, other than those to be determined, occurring in physiological fluids or extracts of organs is unavoidable. In the new apparatus the variable accidental colour admixtures can be easily matched, but only the essential colour value of the reaction itself is taken for the purpose of calculation (see example later). As a final advantage may be claimed the saving in time in preparing, as is usually necessary, a fresh standard solution for comparison. This is replaced by Lovibond's colour units, which are reproducible and permanent. The practical advantages of this colour system are recognised in industrial chemistry, where it has been used extensively since its introduction forty years ago [Lovibond, 1887], but references to its application are scanty in biochemical literature [Palmer and Kempster, 1919; Calvert, 1923; Baker and Marrian, 1927]. The accuracy of the colour units may be controlled spectrophotometrically [Gibson, Harris and Priest, 1927].

Although all the evidence so far available [Rosenheim and Webster, 1926, 1927; Willimott and Wokes, 1927; Accessory Food Factors Committee, 1927] is in favour of the specificity for vitamin A of the $AsCl_3$ (or $SbCl_3$) colour reaction, it seems advisable to await still further confirmation before recommending the colorimetric test for general use as a reliable substitute for the animal experiment. This caution seems to be the more necessary, in that the ease with which estimations can be carried out with the new colorimeter might encourage manufacturers, who have no facilities for controlling the results biologically, to rely on the colorimetric test exclusively.

In order to correlate the colorimetric with the biological test, it was decided after considerable experimental work to take 20 mg. of cod-liver oil as the standard amount for analysis. It was found that the daily administration of this quantity of a good average cod-liver oil, which served as a standard, was the minimum amount required to restore normal growth, *i.e.* to induce a weekly increase of 8–10 g. in rats kept on a diet free from vitamin A. The same amount of this oil equalled 10 blue standard units when mixed with 1 cc. of the reagent, and measured in a layer of 10 mm. thickness. As a preliminary indication, therefore, of the value of an unknown oil in vitamin A, it may be taken that 10 blue standard units correspond to one "rat unit" of vitamin A when the colorimetric comparison is made under the indicated conditions.

The following procedure, the outcome of two years' experience, is recommended for carrying out the test.

(*a*) *Arsenic chloride test.* The oil is measured by means of a Wright's capillary micro-pipette [Wright and Colebrook, 1921], graduated with mercury to hold 25 $mm.^3$ and delivering 20 mg. oil into a test-tube of clear white glass of 10 mm. internal diameter. 1 cc. of the reagent (pure $AsCl_3$) is delivered from a standard 10 cc. burette into the test-tube and a reading taken in the colorimeter immediately (time limit 30 seconds). The results are expressed in standard units of blue. Five consecutive tests are made in each case, differing on the average by ± 0·10 unit.

(*b*) *Antimony chloride test.* By means of a standard pipette 1 cc. of the oil is measured into a measuring flask of 5 cc. capacity. The pipette is rinsed out three times with chloroform, and the solution made up to volume with the same solvent. In order to make the results comparable with the $AsCl_3$ and the biological test, 0·2 cc. (= 40 mm.^{3} oil) of the solution is measured into a test-tube (10 mm. diameter) and mixed with 2 cc. of a 30 % antimony chloride solution in chloroform, delivered from a standard burette. Readings are taken immediately as above, but, owing to the slightly increased stability of the $SbCl_3$ reaction, a longer time limit is generally permissible, readings taken within 1 minute showing no greater difference than ± 0·10 unit as the average of five consecutive estimations. Results obtained on the same oils by different observers at different times agreed within ± 0·50 unit, showing the usual variations due to differences in colour perception of the observers.

Identical values are obtained by the use of either $AsCl_3$ or $SbCl_3$ as a reagent for the colour production. The latter reagent is conveniently prepared by dissolving the required amount of antimony trichloride (B.D.H.) in ordinary B.P. chloroform by rapidly warming to about 50° on a water-bath. A perfectly clear solution results, which keeps unchanged when stored in well-stoppered bottles. A stock solution, prepared 12 months ago and kept in a clear glass bottle under ordinary laboratory conditions, gave constant results when tested at intervals on the same oil. Since such a solution is more convenient in use than undiluted $AsCl_3$, it is preferable for general work, except in the rare cases where an extremely low result requires confirmation by the $AsCl_3$ reagent [Rosenheim and Webster, 1927; see also Wilson, 1927]. This lack of sensitiveness in the antimony reagent is clearly due to the dilution with chloroform of both the oil and the reagent. The explanation suggested by Wokes and Willimott [1927, 1] that a separation of the chloroform solution into two layers may have been the cause of the discrepancy observed is unwarranted, since such has never occurred in our experience when the solution is prepared and kept as above described. It might be pointed out in view of the attempts to increase the accuracy of the reaction [Wokes and Willimott, 1927, 2] that this need is not apparent, since the error of the colorimetric test is out of all proportion smaller than that of the biological animal experiment, which may be as much as 40 % with the technique at present in use.

Further applications. In applying the instrument to any of the colorimetric methods in use, it is only necessary to construct a reference curve by plotting on the ordinate the number of colour units, determined by the estimation of the colour value of solutions containing known amounts of the substance to be analysed, the latter being plotted on the abscissa. As an illustration may be given the curve (Fig. 2) obtained for cystine by the use of the phosphotungstic reagent of Folin and Looney [1922]. I am indebted to Mr W. K. Anslow for the construction of this curve.

The accuracy of the method is proved by the analysis of a solution containing 0·19 % cystine, the amount being unknown to the observer. The result was 0·19 % by the colorimetric method and by calculation from the sulphur content.

It may further be suggested that the apparatus might be easily adapted in routine work to colorimetric p_H determinations, the colour values of various indicators expressed in Lovibond's standard units, covering a p_H range of 1·2–9·0, being available [Taub, 1927].

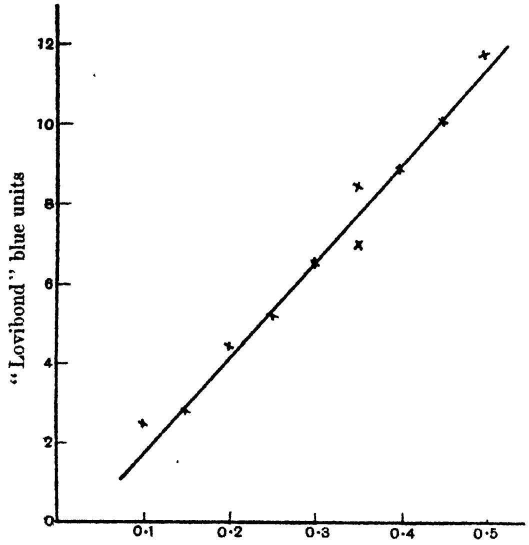

Fig. 2.

Addendum. By Edgar Schuster.

The apparatus consists of a wooden box, on the front of which is mounted a metal plate, painted a dead black. This plate with some of the surrounding woodwork is shown in the photograph (Fig. 1). Near its upper edge is a circular recess in which are two windows. A blackened tube with an eye-piece (not shown) fits this recess and serves to exclude extraneous light. The upper window is for viewing the coloured solution to be tested, and the lower one for the combination of coloured glasses with which its colour is matched. The liquid to be tested is contained in a standard test-tube (or cell) which is supported in a mounting so that its lower part comes between the upper window and the source of light. The top of the test-tube may be seen in the photograph. Behind the lower window are seven horizontal frames supported in a rack as closely as possible one behind the other. In the first frame are nine glasses, which, in combination with a blank space in the frame, provide a series of ten degrees of blueness differing by whole units: *i.e.* 0, 1, 2, 3, 4, 5, 6, 7, 8, 9. In the second frame the glasses form a scale of blueness rising by increments of one-tenth of a unit from 0 to 0·9. The third and fourth frames give units and tenths of units respectively of yellow, and the fifth and sixth frames, of red. The seventh frame holds a blank space and three glasses, each having a tint of 10 units, and being blue, yellow and red respectively. Lateral movement of the frames is controlled by the seven knobs, which can be slid to and fro in the seven slots seen in the photograph between the circular recess and the bottom of the plate. The two upper knobs are coloured blue, and control the first and second frames. Between them is a scale, by which the tint of the glasses appearing behind the window may be read. As shown in the photograph the upper knob is opposite the 1 on this scale, and the lower knob, the 2. This means that the glasses shown are the 1 unit blue and the 0·2 unit blue, giving in combination 1·2 units of blueness. The next two knobs are coloured yellow and control the frames containing the yellow glasses, and the fifth and sixth knobs are coloured red and operate the red glasses. Scales similar to that between the two blue knobs are fitted between the two yellow and the two red. The lowest knob is black and moves the seventh frame; whether one of the three glasses or the blank space is showing is indicated by the row of coloured discs—white, blue, yellow or red which are fixed above the slot.

The mechanical arrangement by which the knobs control the movement of the frames is as follows: each knob is screwed to the middle of a long strip of steel, lying in the inside of the apparatus and supported horizontally by two guides carried on vertical steel bars. A descending member of the correct length is fixed to each end of each frame and its lower end is connected with the corresponding horizontal strip by a suitable distance piece. It is obvious that the number of frames and of standard glasses carried in each may be modified to suit special requirements.

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CLXXV. NOTE ON THE INDUCED FLUORESCENCE OF ERGOSTEROL.

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(*Received November 3rd, 1927.*)

A BRILLIANT blue fluorescence is observed when ergosterol is exposed to a source of ultra-violet rays (mercury vapour lamp) from which the visible rays are screened off by "Wood's glass" and a screen of bromine vapour. Eight specimens of different origin were examined, four of which had been prepared from ergot and four from yeast. Under the same conditions pure cholesterol, freed from ergosterol by bromine, showed no fluorescence. The phenomenon stands in no relationship, however, to the formation of vitamin D from ergosterol by ultra-violet irradiation. It has been found that under certain conditions preparations of non-fluorescent ergosterol can be obtained, which are rendered as highly antirachitic by irradiation as the fluorescent ones. Moreover, a specimen of cholesterol which could not be activated and was free from ergosterol, as shown by the spectroscopic test, possessed a strong blue fluorescence. This was traced to a previous treatment with charcoal (norite) during recrystallisation from alcohol, and a similar treatment induced fluorescence in non-fluorescent ergosterol. Since the same result is also produced by repeatedly recrystallising non-fluorescent ergosterol from ordinary ether, kept in light and containing peroxides, the conclusion seems justified that the fluorescence of sterols under the above conditions is due to the formation of traces of oxidation products.

CLXXVI. THE PHYSIOLOGICAL RÔLE OF VITAMIN B.

PART V. THE RELATION OF INANITION TO VITAMIN B DEFICIENCY IN PIGEONS.

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PREFATORY NOTE.

THROUGHOUT this paper vitamin B refers to the accessory food factors present in yeast. We have satisfied ourselves that pigeons fed on a synthetic diet of the type used throughout this work but supplemented with 4 % of yeast extract that has been heated with alkali for 4 hours to destroy the "anti-neuritic" factor behave exactly as do those which receive the unsupplemented ration. The presence of the treated yeast extract, providing amongst other substances the heat-stable factor [Hassan and Drummond, 1927], does not ameliorate the condition, or retard the decline or the onset of nervous symptoms, at any rate in the case of birds feeding voluntarily.

INTRODUCTION.

In previous communications [Drummond and Marrian, 1926; Kon and Drummond, 1927] of this series it has been pointed out that a large proportion of the abnormalities exhibited by animals deprived of vitamin B is due to the inanition that results from the failure of appetite rather than to any specific effect of the deficiency itself.

From the studies of the rat and of the pigeon it appeared that the lowered body temperature and metabolism, the loss of weight and atrophy of most tissues, and the hyperglycaemia could be so regarded, but it was not certain from these studies or the researches of earlier workers how intimately the nervous manifestations and the hypertrophy of the adrenal glands were related to starvation.

The investigation reported in this paper was carried out with the object of throwing light on these two points.

EXPERIMENTAL.

In the first experiment the pigeons were divided into four groups in such a manner that the average weight of each group was as nearly as possible the same. All were deprived of food with the exception that ample supplies of water were available. It must be remembered that simple starvation may be considered as one form of vitamin B deficiency. The birds in groups A and C served to differentiate between the effects of withholding food in presence and absence of vitamin B, in that the latter received every day 10 cc. of an aqueous 10 % solution of yeast extract (marmite) administered directly into the crop by a rubber tube.

In addition there were two other groups, B and D, similar to A and C except that each bird received daily from 100 to 200 cc. of water introduced directly into the crop in two equal doses every morning and evening.

These two groups were studied in order to investigate the claims of Chamberlain, Bloombergh and Kilbourne [1911], Chamberlain and Vedder [1911] and of Eijkman and van Hoogenhuyze [1916] to have produced the characteristic nervous symptoms of vitamin B deficiency in fowls and pigeons by "washing out" the birds with large volumes of water during the course of starvation.

In the second experiment three groups of pigeons were fed on a diet deficient in vitamin B. An artificial diet of the type used by Randoin and Simonnet [1924] was used. The diet was composed as follows:

Rice starch	66 %	Butter	4 %
Caseinogen	16 %	Paper pulp	2 %
Agar-agar	8 %	Salt mixture (McCollum)	4 %

The birds in group F were allowed to feed voluntarily on the deficient diet. Those of group G were forcibly fed with about 20 g. of the diet daily, whereas those of group H were forcibly fed with about 15 g. of the deficient diet and in addition were given daily 1 g. of yeast extract, in order to serve as a control against the diet and the possible ill effects of the forcible feeding. The diet could be readily introduced into the crop in a coarsely powdered form by pouring it down a wide bore glass tube and regulating the rate of flow with a thin glass rod.

All the birds were starved for 24 hours before the "initial weight" was recorded, in order to eliminate errors due to a loaded crop.

General behaviour of the starving birds.

Apart from a steady loss of body weight and an initial fall in body temperature the birds did not display any striking symptoms for the first few days. Later the feathers became ruffled and the birds seemed disinclined for movement of any sort, although they appeared to be quite strong when they desired to walk or fly. The final phase usually appeared with great suddenness and was characterised by rapid onset of muscular weakness and a sharp fall in the body temperature, closely resembling the condition described in rats [Drummond and Marrian, 1926]. This dramatic collapse nearly always occurred in the very early morning hours, and we think it probable that nervous symptoms similar to those which we observed in the birds of all groups have escaped the attention of most investigators in the past because close observation of the animals during the whole 24 hours was not maintained. Perhaps the most characteristic behaviour of the birds in the premortal phase of inanition, whether vitamin B extracts were given or not, was a slow dropping forward of the head followed by a step forward to readjust the balance of the body. This movement would go on rhythmically for several hours, interrupted sometimes by retrograde steps and in certain cases by more or less well-marked head retraction and cartwheel turning. To this last point we will again refer.

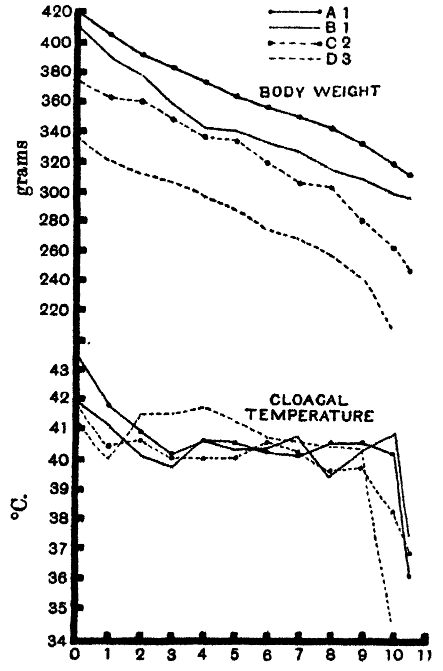

Fig. 1.

In Fig. 1 are drawn curves illustrating the weights and temperatures of birds in groups A–D. It will be seen that the loss of body weight is of the same order in all four groups, and that neither the rate of loss of body weight nor the course of the temperature curves is affected by the administration of amounts of yeast extract sufficient to supply the vitamin B requirements of the pigeon when feeding normally. These observations confirm in detail those that we have recorded for the rat.

General behaviour of the birds on the deficient diet.

For about the first week the birds in group F ate normally and the body weight was maintained. This period was followed by one showing progressively increasing fall in food intake, and a corresponding decrease in body weight. After about 30 days the birds usually showed the typical nervous symptoms of avian "beriberi," but in a few cases the pigeons failed to show any such symptoms and eventually died in a state of extreme weakness. In nearly all cases the birds by the time convulsions were observed had shown a loss of body weight of the same order as that shown by the starving birds, *i.e.* 30 to 40 % of the original weight.

The birds in group G behaved normally for about the first week, and appeared to be able to assimilate all the diet that was administered. Later, however, a small amount of the food was often vomited. A rough quantitative measure of the amount of rejected diet showed that at least 80 % of the diet fed was retained and digested. With no exception, the birds in this group developed violent convulsions within 20 days, at a period when the reduction of body weight was so slight as to be almost negligible.

In order to avoid errors in body weight due to a loaded crop, the food was removed quantitatively after the death of the bird, dried and the weight subtracted from the total weight of the bird before death.

Hypertrophy of the adrenal glands in inanition and vitamin B deficiency.

One of the most striking changes that have been observed in animals suffering from a deficiency of vitamin B is a well-marked hypertrophy of the adrenal glands [McCarrison, 1919, 1920; Kellaway, 1921; Findlay, 1921; Beznak, 1923; Lasowsky and Sumnitzki, 1926]. Changes of a similar order have also frequently been observed as a result of simple starvation [McCarrison, 1919; Vincent and Hollenberg, 1920, 1921; Findlay, 1921; Beznak, 1923]. It must, however, be borne in mind that complete deprivation of food also involves a deficiency of vitamin B, so that the fact that the adrenals hypertrophy as a result of starvation does not warrant the assumption that the enlargement is not specifically due to the deficiency of the accessory factors. Beznak [1923] indeed claims to have shown that no hypertrophy of the adrenal glands is exhibited by starving pigeons that are receiving ample supplies of vitamin B in the form of yeast extract.

In nearly all cases, the starving birds were killed during the final collapse which we have described previously. The vitamin B-deficient birds were killed immediately the first signs of convulsions appeared. Immediately after death the glands were dissected out and weighed. In a number of cases estimations of the adrenaline present were carried out by a modification of the Folin, Cannon and Denis method [Baker and Marrian, 1927]. Table I shows the results obtained from a number of normally fed pigeons.

Table I. *Normal pigeons starved 24 hours before experiment.*

Weight (g.)	Sex	Weight of adrenals (g.)	Weight of adrenals per kg. body weight	Adrenaline (mg.)	Adrenaline per 1 g. gland (mg.)	Adrenaline per kg. body weight (mg.)
372	♀	0·0195	0·0524	0·055	2·82	0·148
263	,,	0·0317	0·1206	0·106	3·34	0·403
331	,,	0·0196	0·0592	0·048	2·45	0·145
388	,,	0·0170	0·0438	0·045	2·65	0·116
334	,,	0·0191	0·0572	0·056	2·93	0·167
296	,,	0·0192	0·0648	0·044	2·29	0·148
326	,,	0·0270	0·0828	0·072	2·66	0·214
Average 330	—	0·0219	0·0687	0·061	2·73	0·191
320	♂	0·0244	0·0769	0·066	2·71	0·206
395	,,	0·0216	0·0547	0·067	3·08	0·169
309	,,	0·0270	0·0879	0·069	2·55	0·223
304	,,	0·0319	0·1049	—	—	—
386	,,	0·0211	0·0546	0·076	3·60	0·197
322	,,	0·0154	0·0478	0·052	3·37	0·161
344	,,	0·0302	0·0878	0·072	2·38	0·209
Average 340	—	0·0245	0·0735	0·067	2·95	0·194

It is evident from the figures in Table II that the adrenal gland is hypertrophied in the later stages of starvation and that this enlargement is not prevented by the administration of extracts rich in vitamin B. This is contrary to the conclusion reached by Beznak [1923], and we are unable to account for his results.

The birds in group F which were voluntarily feeding on the deficient diet also showed with one exception a definite but rather smaller hypertrophy (Table III). At first sight it might have appeared that this could be ascribed entirely to the complicating effects of inanition. However, the forcibly fed birds in group G, which only showed a very slight decrease in body weight, exhibited an enlargement of the glands of the same order as that shown by the birds in group F. The birds in group H showed no abnormalities of the adrenal glands. Both the weight of the glands and the adrenaline estimated were of the same order as for the normally fed pigeons.

There is considerable divergence of opinion as to whether the hypertrophy of the glands observed in vitamin B deficiency and starvation is medullary or cortical in origin. As a result of chemical determinations of the adrenaline content of the glands McCarrison [1920] is convinced that the hypertrophy is medullary. On the other hand Beznak [1923], adopting similar methods, could find no increase in the adrenaline content of the glands and concluded that the cortex alone hypertrophies. As a result of histological studies of the adrenals of pigeons fed on polished rice, Findlay [1921] and Lasowsky and Sumnitzki [1926] also concluded that the hypertrophy is cortical. Indeed Findlay claims to have shown that after curing a pigeon with yeast extract there is an immediate disappearance of a large amount of lipoid material from the cortex.

Our own results show that in all cases the total adrenaline present in the starving birds is considerably increased. In some instances this increase is proportional to the hypertrophy of the glands, from which it would seem

Table II.

Group	No. of bird	Sex	Initial weight (g.)	Final weight (g.)	Initial wt / Final wt	Weight of adrenals (g.)	Weight of adrenals per kg. body weight		Total adrenaline present (mg.)	Adrenaline per 1 g. gland (mg.)	Adrenaline per kg. body weight		Days starving	Remarks
							Initial (g.)	Final (g.)			Initial (mg.)	Final (mg.)		
A	38	♀	280	174	1·61	0·0388	0·1385	0·2229	—	—	—	—	11	Complete collapse
	35	„	320	193	1·66	0·0374	0·1169	0·1938	0·108	2·88	0·337	0·559	12	
	2	„	358	242	1·48	0·0275	0·0768	0·1136	—	—	—	—	10½	Quite strong and steady
	3	„	330	215	1·54	0·0602	0·1824	0·2800	—	—	—	—	10½	Complete collapse. Slight head retraction just before death
	4	„	325	228	1·42	0·0457	0·1407	0·2005	—	—	—	—	7½	Not very weak. Loss of equilibrium after swinging
	5	„	295	191	1·54	0·0329	0·1115	0·1722	—	—	—	—	10	Cartwheels and head retraction
Average		♀	318	207	1·54	0·0404	0·1278	0·1972	—	—	—	—		
	33	♂	380	226	1·68	0·0530	0·1394	0·2345	0·117	2·21	0·308	0·518	13½	Very weak. Slight head retraction just before death
	1	„	405	310	1·31	0·0274	0·0676	0·0884	—	—	—	—	10½	Quite strong and steady
	6	„	328	210	1·56	0·0610	0·1859	0·2905	—	—	—	—	8½	Loss of equilibrium after swinging
Average		♂	371	249	1·52	0·0471	0·1310	0·2045	—	—	—	—		
B	3	♀	323	220	1·47	0·0193	0·0590	0·0880	—	—	—	—	10½	Quite strong and steady
	1	♂	390	295	1·32	0·0455	0·1166	0·1542	—	—	—	—	10½	Quite strong and steady
	2	„	364	253	1·44	0·0360	0·0989	0·1423	—	—	—	—	9½	Very weak. Cartwheels and head retraction
	4	„	330	227	1·45	0·0384	0·1164	0·1691	—	—	—	—	8	Complete collapse
	6	„	275	199	1·38	0·0530	0·1927	0·2660	—	—	—	—	6	Loss of equilibrium after swinging and head retraction
Average		♂	340	244	1·39	0·0432	0·1312	0·1829	—	—	—	—		
C	42	♀	310	208	1·49	0·0320	0·1032	0·1490	0·100	3·12	0·322	0·480	11	Very weak
	43	„	330	211	1·56	0·0451	0·1366	0·2157	0·096	2·13	0·291	0·455	17	Weak
	1	„	315	205	1·51	0·0448	0·1422	0·2185	—	—	—	—	7½	Loss of equilibrium after swinging
	4	„	352	247	1·43	0·0480	0·1362	0·1943	—	—	—	—	10½	Disturbed by swinging. Head retraction and convulsions just before death
	5	„	323	229	1·41	0·0512	0·1585	0·2236	—	—	—	—	9½	Very weak. Head retraction for short periods
	6	„	286	200	1·43	0·0442	0·1545	0·2210	—	—	—	—	6½	Loss of equilibrium after swinging
Average		♀	319	217	1·47	0·0442	0·1385	0·2037	0·098	2·63	0·307	0·467		
	41	♂	340	210	1·62	0·0692	0·2353	0·3295	0·107	1·84	0·315	0·509	13	Marked head retraction. A tendency to turn cartwheels
	40	„	330	207	1·59	0·0436	0·1321	0 2106	0·104	2·39	0·315	0·502	14½	Very weak
	39	„	360	213	1·69	0·0577	0·1603	0 2709	0·099	1·72	0·275	0·465	15	
	2	„	363	246	1·47	0·0428	0·1179	0·1739	—	—	—	—	10½	Quite strong and steady
Average		♂	348	219	1·59	0·0533	0·1614	0·2462	0·103	1·88	0·302	0·492		
D	3	♀	320	213	1·50	0·0444	0·1387	0·2084	—	—	—	—	10	Very weak
	6	„	291	210	1·39	0 0449	0·1543	0·2138	—	—	—	—	8	Very weak. Slight tendency to head retraction
Average		♀	306	212	1·44	0·0447	0·1465	0·2111	—	—	—	—		
	1	♂	375	274	1·36	0·0479	0·1277	0·1748	—	—	—	—		
	2	„	351	276	1·27	0·1141	0·2350	0·4144	—	—	—	—	7½	Cartwheels and head retraction

reasonable to suppose that in such cases the hypertrophy is medullary. In other cases, however, low figures for the adrenaline per g. of the gland were obtained although the total adrenaline present was above normal. This may either represent an approach to exhaustion of the adrenaline reserves of the gland in the last stages of inanition, or else an additional hypertrophy of the cortex.

The results obtained on the forcibly fed pigeons are of considerable interest, since in every case the total adrenaline present was either normal or slightly low, and in every case the adrenaline per g. of gland was definitely low (Table III). Since all these birds were killed at the first sign of convulsive symptoms, and at a time when they were muscularly strong, it does not seem possible that an exhaustion of the adrenaline in the gland had occurred. A possible explanation of these results would be to suppose that in "beriberi" uncomplicated by the effects of inanition only the cortex undergoes hypertrophy, and that in starvation, and "beriberi" associated with starvation, the hypertrophy is mainly medullary, but no decision can be reached without further experiments.

Blood-sugar of pigeons during inanition.

It is a clearly established fact that pigeons showing typical symptoms of "beriberi" may often show a distinct hyperglycaemia [Funk and Schönborn, 1914; Funk, 1920; Randoin and Lelesz, 1925; Collazo, 1923]. Kon and Drummond [1927] carefully controlled a series of pigeons against the complicating factors of starvation by feeding a second group of birds with the same amounts of food as were being voluntarily consumed by those receiving the deficient diet, at the same time administering excess of vitamin B in the form of yeast extract. The blood-sugars of these two groups were identical and they concluded that the hyperglycaemia observed during vitamin B deficiency is wholly attributable to inanition.

Blood-sugar determinations carried out on samples removed from the wing vein on our own two groups of starving pigeons (A and C) tend to support this conclusion (Table IV) and clearly show that the feeding of yeast extract during starvation had no effect on the blood-sugar. Except in the last stages of inanition both groups show a slight but distinct hyperglycaemia compared with the control group of normally fed birds.

The normal control birds were starved for 24 hours previous to the determinations in order that the blood-sugar should fall to the resting level.

A series of estimations of blood-sugar made during the course of the experiment shows that the slight hyperglycaemia which appears after the first day or two of deprivation of food and which persists for several days is followed by a marked hypoglycaemia (Fig. 2). The fall in the concentration of blood-sugar occurs very suddenly and is contemporary with the fall of temperature which characterises the pre-mortal phase. This picture is similar in all respects to that we have described in the case of the rat [Drummond and Marrian, 1926].

Table III.

Group	No. of bird	Sex	Initial weight (g.)	Final weight (g.)	Initial wt / Final wt	Weight o adrenals (g.)	Weight of adrenals per kg. body weight		Total adrenaline (mg.)	Adrenaline per 1 g. gland (mg.)	Adrenaline per kg. body weight		No. of days on diet
							Initial (g.)	Final (g.)			Initial (mg.)	Final (mg.)	
F*	328	♀	303	208	1·45	0·0516	0·1703	0·2481	0·142	2·75	0·468	0·682	33
	329	♂	357	233	1·53	0·0414	0·1159	0·1776	0·093	2·25	0·261	0·399	26
	326	,,	349	250	1·39	0·0192	0·0550	0·0768	0·041	2·13	0·117	0·164	27
	39	,,	452	362	1·24	0·0446	0·0986	0·1232	0·075	1·68	0·166	0·207	29
	32	,,	387	286	1·35	0·0395	0·1020	0·1381	0·084	2·13	0·217	0·293	30
	46	,,	389	285	1·34	0·0434	0·1116	0·1523	0·139	3·20	0·357	0·488	34
Average		♂	387	283	1·37	0·0376	0·0972	0·1328	0·086	2·28	0·224	0·310	
G*	839	♀	301	245	1·23	0·0370	0·1229	0·1510	0·039	1·05	0·129	0·159	19
	3302	♂	411	406	1·01	0·0376	0·0915	0·0926	0·069	1·83	0·168	0·169	15
	718	,,	352	321	1·09	0·0546	0·1551	0·1701	0·072	1·32	0·204	0·224	13
	749	,,	394	390	1·01	0·0519	0·1317	0·1331	0·068	1·31	0·172	0·174	12
	25	,,	347	326	1·07	0·0400	0·1149	0·1227	0·038	0·95	0·109	0·117	16
Average		♂	376	361	1·05	0·0460	0·1233	0·1296	0·062	1·35	0·163	0·171	
H	994	♀	337	364	0·93	0·0284	0·0843	0·0780	0·062	2·18	0·184	0·170	17
	734	,,	332	357	0·93	0·0276	0·0831	0·0773	0·058	2·10	0·175	0·163	17
Average		♀	335	361	0·93	0·0280	0·0837	0·0777	0·060	2·14	0·180	0·167	
	872	♂	278	254	1·09	0·0260	0·0934	0·1024	0·070	2·69	0·252	0·275	17
	924	,,	317	323	0·98	0·0236	0·0744	0·0731	0·075	3·18	0·237	0·232	17
Average		♂	298	289	1·04	0·0248	0·0839	0·0878	0·073	2·94	0·245	0·254	

* All the pigeons in groups F and G showed typical convulsions.

We are inclined to attach importance to the fact that all birds showing head-retraction simulating "beriberi" convulsions were passing through this pre-mortal phase.

Table IV.

	Normal. Blood-sugar %	Group A. Starving 4–9 days. Blood-sugar %	Group C. Starving 4–9 days. 1 g. yeast extract daily. Blood-sugar %
	0·174	A 1 0·174	C 1 0·206
	0·176	A 2 0·202	C 2 0·160
	0·186	0·210	C 3 0·242
	0·190	A 3 0·224	C 4 0·234
	0·178	0·242	C 5 0·186
	0·191	A 4 0·214	C 6 0·236
	0·171	A 5 0·186	C 39 0·209
	0·183	A 6 0·210	C 40 0·202
	—	0·226	—
	—	A 38 0·215	—
	—	A 33 0·202	—
Average	0·181 %	0·209 %	0·209 %

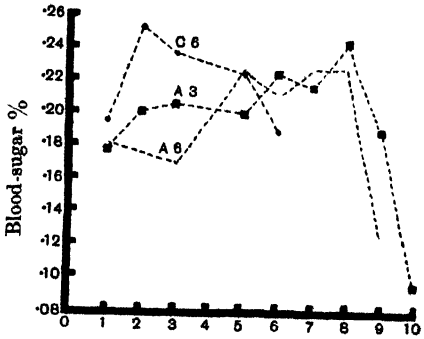

Fig. 2.

Head-retraction in pigeons during inanition.

Chamberlain, Bloombergh and Kilbourne [1911] and Chamberlain and Vedder [1911] reported that they were able to produce the typical nervous symptoms of "beriberi" in birds by "washing out" the tissues by administering large volumes of water during starvation. These results were confirmed by Eijkman and van Hoogenhuyze [1916] who showed that these symptoms could be cured by the administration of vitamin B notwithstanding starvation, and concluded that "polyneuritis" occurring as a result of feeding on a vitamin B-deficient diet cannot be caused by inanition.

It will be observed on referring to Table II that in each of the two groups A and B there were several cases of pigeons that had quite definite head-retraction. Pigeons A 5 and B 2 displayed extremely well-marked "polyneuritic" symptoms. In addition to showing a definite head-retraction, these birds turned "cartwheels"; their general appearance being indistinguishable from that of those suffering from true vitamin B deficiency. In many other instances less marked but quite definite head-retraction was observed. By disturbing the equilibrium of all the birds these symptoms could be accentuated. It must be pointed out that these symptoms only occurred during the final pre-mortal phase, and that they were of considerably shorter duration than those observed in vitamin B deficiency.

The appearance of these typical "beriberi"-like symptoms in several of the birds in group A seems to indicate that flushing with water is an unnecessary procedure.

We were surprised to observe several cases of head-retraction in groups C and D which were receiving yeast extract daily. Pigeons C 4, C 5 and D 6, D 2 all showed slight but definite head-retraction for a short period before death, while C 41 and D 1 displayed more definite symptoms which were quite as well marked as any in groups A and B.

As these birds had been receiving yeast extract every day and had been given a dose within a few hours of the appearance of the convulsions, we have found it hard to reconcile our observations with those of Eijkman and van Hoogenhuyze that the convulsions exhibited by starving birds may be cured by administration of vitamin B[1].

Discussion.

Before we had carried out the experiments on the pigeons that received the food deficient in vitamin B forcibly we were convinced that the hypertrophy of the adrenals in ordinary vitamin B deficiency was due merely to inanition and was unrelated specifically to the absence of the vitamin. Obviously this view could be held no longer when enlarged glands were exhibited by the birds whose body weight had been more or less satisfactorily maintained by forcible administration of the food deficient in vitamin B. Moreover, the fact that the control birds, which received in addition to their ration of food a supplement providing an ample supply of vitamin B showed normal glands ruled out the possible explanation that hypertrophy might be caused by the actual process of forcible feeding.

As we have pointed out, our results could be explained on the rather improbable hypothesis that inanition produces medullary hypertrophy whilst deficiency of the factor B tends to cause enlargement of the cortical tissue.

We prefer to make no further comment at this stage of the enquiry on the results we have obtained. Further work is in progress which may throw light on the curious facts we have observed.

Regarding the appearance in the starving birds of convulsive seizures similar in general character to those exhibited by birds fed on diets deficient in vitamin B, it must be admitted that when the case of those which received a daily ration of yeast extract is considered the whole question becomes a much broader one than was originally raised by the experimental results of Chamberlain and his colleagues and Eijkman and van Hoogenhuyze.

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[1] Dr R. R. Williams very kindly placed the manuscript of his paper [1927] in our hands whilst we were preparing the results of our work for publication. It is interesting that he noted that birds which exhibited nerve symptoms on diets containing ample supplies of vitamin B had lost from 30 to 40 % of their original body weight, a loss of the order of that shown by our starving birds at the time of onset of symptoms.

In the first place we are not entirely satisfied that the symptoms shown by the birds "flushed" with water by Chamberlain were due to the washing out of vitamin B from the tissues as they suggested, because in our experience there was just as marked a tendency for the convulsions to occur in birds whose consumption of water was limited to the very small quantity they drank voluntarily. Secondly, we find it difficult to reconcile our observations with the statement of Eijkman and van Hoogenhuyze that they could cure "polyneuritis" in starving water-flushed birds with extracts of vitamin B.

We have described the onset of typical "beriberi" symptoms in starving pigeons which have been receiving daily an ample dose of vitamin B, and somewhat similar observations are recorded by Williams [1927] (see footnote to p. 1345).

The problem that faces us is not only to reconcile these conflicting views, but to provide an explanation of the curious fact that whereas pigeons feeding voluntarily on a ration deficient in vitamin B will usually show "beriberi" symptoms at about the 30th day, those forcibly fed on this diet do so at about the 16th day, and those deprived of food altogether at about the 9th.

We do not wish to commit ourselves to any definite opinion on these obviously difficult questions at present. For the time being we are, as we have already remarked, inclined to attach importance to the fact that whilst the nervous manifestations in the "beriberi" pigeon that is receiving food often appear at a time when the body temperature is not markedly subnormal and when the sugar concentration in the blood is usually slightly above the normal level, we have never observed the convulsions in starving birds except in the pre-mortal phase when the blood-sugar is very greatly reduced and the temperature is falling rapidly.

This difference raises in our minds the query whether the changes in the nervous system that are responsible for the convulsions may not be brought about by a variety of agents. This view is strengthened by reference to the work of Hess, which we have ourselves confirmed, and which showed that a condition closely simulating "beriberi" in pigeons can be produced by sublethal doses of cyanides. If this view be regarded as reasonable it attracts attention to the conception, many times expressed in the literature of experimental beriberi, that in vitamin B deficiency the underlying cause of the convulsions is a toxic substance, of which either the production is suppressed or the poisonous action is neutralised when vitamin B is administered. Dr Williams strikes the same note in the concluding paragraph of his discussion [1927, p. 1351].

In the investigations we are now reporting a considerable amount of additional evidence was obtained from the histological examination of the peripheral nervous system of the pigeons. It all served to confirm the opinion previously expressed [Woollard, 1927] that the minor changes observed are attributable solely to inanition and are in no way directly related to vitamin B deficiency.

SUMMARY.

(1) The administration of extracts rich in vitamin B does not prevent the hypertrophy of the adrenal glands in pigeons that are deprived of food. This is contrary to the conclusion of Beznak.

(2) In most cases the adrenaline content of the glands of starving pigeons, whether receiving vitamin B or not, is increased proportionately to the hypertrophy. This tends to confirm the opinion of McCarrison that the hypertrophy is medullary.

(3) A slight but definite hyperglycaemia is observed in pigeons during most of the course of inanition. It is followed by a marked fall in the amount of blood-sugar coincident with the pre-mortal fall in body temperature. The administration of extracts rich in vitamin B has no effect on the blood-sugar levels of such birds.

(4) Nervous symptoms similar to those observed in pigeons suffering from vitamin B deficiency were observed in a number of starving birds. The incidence of such symptoms was not increased by the daily administration of large volumes of water.

These convulsions cannot be due to a vitamin B deficiency resulting from starvation since several cases were observed in birds receiving daily administration of vitamin B.

(5) The symptoms observed in starving birds always occurred during the period just before death when body temperature and blood-sugar were much reduced. This is in contrast to the established fact that nervous manifestations in pigeons nourished on rations deficient in vitamin B usually appear when the temperature is but slightly reduced and the blood-sugar level is, if anything, above normal.

(6) By forcibly feeding pigeons on larger quantities of a diet deficient in vitamin B than they would voluntarily consume, typical nervous symptoms of beriberi were produced in about 16 days, at a time when the loss of body weight was almost negligible.

(7) The adrenal glands of the forcibly fed pigeons were hypertrophied to a considerable extent, although no corresponding increase of the adrenaline content was observed. At present no satisfactory explanation of this is forthcoming.

We wish to express our sincere gratitude to Prof. Bertrand for providing us with laboratory accommodation at the Pasteur Institute during part of this investigation.

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CLXXVII. A NOTE ON THE EFFECTS ON PIGEONS OF AN EXCLUSIVE DIET OF RICE MEAL, BRAN AND POLISH.

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(*Received October 26th, 1927.*)

Moore [1914] has described a condition in swine closely resembling beriberi, which, however, ensued from diets consisting largely of the outer coats of rice known as rice meal[1]. This product is, of course, rich in its content of anti-beriberi vitamin. This led the writer to test the effects on pigeons of exclusive diets of the several commercial by-products of rice milling. The products tested were rice bran, a coarse material consisting largely of the outer bran coats, rice meal, which is the material next removed from the grain in the milling process and rice polish, a fine relatively light coloured product containing a large percentage of starch as well as the last portions of the bran coats. A mixture of the products was also made in the proportion of 14 of bran to 10 of meal to 3 of polish, which is approximately the proportions in which the three products are obtained from the grain.

Exp. 1. It was desired first to prove the presence of adequate amounts of anti-beriberi vitamin in each of the several products mentioned above. Accordingly, four groups of four pigeons each were fed on polished rice *ad lib.*[2]. In addition each bird in one group was fed daily by hand 3 g. of rice polish; in another 3 g. of the meal; in the third the same quantity of bran, and in the fourth an equal amount of the mixture of the four products. The birds were weighed twice a week. All maintained their initial weights or made slight gains and continued to show a healthy and vigorous condition at the end of 60 days when the experiment was discontinued.

Exp. 2. A group of four pigeons was fed *ad lib.* on an exclusive diet of rice bran in the form of a stiff mash which was freshly prepared each day. No. 471 died after 32 days, having lost 49 % in weight; No. 472, at the end of 22 days with a loss of 42 % and No. 473 on the 22nd day having lost 41 %. The symptoms in these birds were not very characteristic, consisting of loss of appetite, a mild diarrhoea and steady decline in strength and weight. On examination of the sciatic nerves after staining by the Marchi method, a distinct fatty degeneration was readily detected in the fibres of all. No. 470

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[1] Mr Moore in a private communication has verified this interpretation of the term rice meal and kindly submitted a small sample for inspection.

[2] Water was furnished from the Washington City water supply in all the feeding experiments described in this paper.

in the course of 40 days lost 26 % in weight, after which its weight remained stationary for an additional 80 days. At the end of this time the bird was killed, having exhibited no characteristic symptoms of disease. On examination, the fibres of the sciatic nerve were found to be extensively degenerated.

Exp. 3. An experiment precisely similar to the above was conducted, using rice meal instead of bran. All four birds lived for 120 days and, except for two instances of temporary decline, maintained their initial weights. At the end of this time, they were killed. The sciatic nerve fibres in all these birds appeared normal.

Exp. 4. Rice polish was fed under the same conditions to four pigeons. All four birds declined slightly during the second or third week but later recovered the loss in large part and lived throughout the 120 days of the experiment. No. 477 remained stationary at about 85 % of its initial weight throughout the latter half of the feeding period, but the remainder maintained approximately their initial weights and all were free from serious outward symptoms of disease. The fibres of the sciatic nerves of birds 475 and 476 showed no lesions, but those of 477 and 478 were found to be moderately degenerated.

Exp. 5. A mixture of 14 parts of bran, 10 of meal and 3 of polish was fed to four birds under similar conditions. No. 467 slowly declined to 64 % of its original weight and on the 65th day was found in a condition of complete paralysis, with severe neck retraction precisely similar in appearance to the condition of polyneuritis induced by feeding exclusively on white rice. Death followed on the same day. No. 468 declined to 76 % of its initial weight but lived for 120 days at this lower level without other evidence of disease. Nos. 466 and 469 retained weight and strength throughout the feeding period. After 120 days the birds remaining were killed as in the previous experiment. The sciatic nerve fibres of No. 467 were slightly degenerated but those of the remainder betrayed nothing abnormal.

Exp. 6. Six pigeons were fed in the same manner on another sample of commercial rice polish which, however, was identical with the first in appearance. These birds all declined fairly rapidly, losing from 30 to 40 % in weight. Nos. 328 and 329 died in 23 days; No. 330 in 10 days; No. 331 in 36 days; No. 332 in 13 days, and No. 333 in 15 days. No. 328 showed a slight ataxia and 333 a moderate neck retraction on the day previous to death. Degeneration of the sciatic nerve fibres was fairly pronounced in 328; slight in the remainder, being especially slight in 333.

It seems evident from the above experiments that rice mill by-products are distinctly harmful to pigeons when fed as the exclusive diet. Apparently there is a great variation in the harmfulness of different samples, an observation which is in entire accord with that of Moore as to the effects of rice meal on swine. It also seems likely that pigeons are more resistant than swine to the harmful factor, although there appear to be extremely wide individual variations in pigeons in this respect.

McCollum, Simmonds and Pitz [1916] have recorded evidence of the presence of a toxic substance in wheat germ and Hart *et al.* [1916] have noted a nervous disorder resembling beriberi in swine, ensuing from a diet rich in wheat products. Rommel and Vedder have also called attention to the marked resemblance between so-called cotton-seed poisoning in swine and human beriberi. That the condition resulting from excessive feeding of cotton-seed is in reality due to poisoning seems to rest on excellent evidence. Taking all these facts into consideration, the most plausible hypothesis by which to account for the harmful effects of large amounts of rice offal on pigeons and swine is that there is present in the outer coats of rice a variable quantity of a more or less distinctly toxic substance. This hypothesis should be tested by further experiment.

These considerations are of especial interest in the suggestions they offer regarding the nature of the "deficiency diseases." How does it happen that what appears to be essentially the same type of nerve lesions is found in beriberi, pellagra and in a mild form in scurvy [McCollum, Simmonds and Pitz, 1916] as well as in animals fed largely on wheat germ, cotton-seed meal or rice offal? Not only are these nerve lesions found in dietary disorders but they have been reliably reported as resulting from oxalic acid [Mauer, 1907], alcoholic and arsenical poisoning [Ross, 1900, 1902].

The writer believes that these facts constitute evidence sufficient to demand consideration that in the "deficiency diseases" there exist toxaemias consequent or incident to dietary disturbances which may play no small rôle in determining the pathology and symptomatology of these diseases. This does not necessarily imply that the foods productive of these diseases are toxic in the ordinary sense. Nor is the opposing argument, that in the case of beriberi the hypothetical toxaemia is corrected by addition to rather than by a subtraction from the diet, necessarily contrary to the view suggested. Hart *et al.* [1916] have shown that effects of the toxic element in wheat which are serious when the diet is otherwise poor disappear almost completely when other dietary defects are corrected.

My thanks are due to Mr G. M. Rommel of the Bureau of Animal Industry, U.S. Dept. of Agriculture, for furnishing part of the necessary rice mill products and also to Lieut.-Col. E. B. Vedder, M.C., U.S.A., for assistance and verification of some of the histological findings.

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CLXXVIII. THE ABSORPTION OF WATER BY GELATIN.

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(*Received October 19th, 1927.*)

During the last half-century a large number of papers has been published on the absorption of water by gelatin and the influence on this process of various physical and chemical factors. A review of the literature has recently been given by one of us [Jordan Lloyd, 1926]. Previous work has shown that acids, alkalis, salts, temperature and the relative masses of the gelatin and the water [Jordan Lloyd, 1920; Kuntzel, 1926] all affect the final water content of a gelatin jelly in equilibrium with its surrounding fluid. The correlation between one factor and another is still, however, a fruitful subject for investigation, and this paper provides such a study in the case of a simple system in which all the diffusible ions are univalent. This system is gelatin in the presence of hydrochloric acid, sodium hydroxide, and sodium chloride, over a temperature range from 0° to 24°, a p_H range of 0·5 to 12, and a concentration of sodium chloride from zero to 2·0 mol. per litre. A uniform relation of 100 cc. of fluid to every 0·1 g. of gelatin was used throughout the experiments.

Throughout this paper the term "gel" is used to denote undispersed gelatin, and "jelly" the characteristic 2-phased rigid solution of gelatin in water.

MATERIAL.

The gelatin used was prepared from Coignet's "Gold Label" leaf gelatin. Twelve sheets were soaked in 3 litres of 0·2 M sodium chloride, adjusted to p_H 3 by the addition of hydrochloric acid; the gelatin was kept in gentle movement, and after 3 or 4 hours the reaction of the fluid was tested and brought back to p_H 3 by the addition of more acid, this process being repeated until equilibrium had been attained. At this stage the whole of the fluid was poured off and replaced by a fresh solution of sodium chloride at p_H 3. This remained on the gelatin overnight, after which a test for calcium always gave a negative result. The gelatin was then dialysed against a stream of distilled water until free from chlorides. The whole preparation lasted a week. During the last stages of washing the gelatin became white and opaque. The dialysed pieces were dried on coarse net at room temperature. The ash content of the purified material varied from 0·05 to 0·02 % of the dry weight at 100°. This method of purification, which is a modification of one used by Smith [1922], is not so rapid, or so complete, as electrodialysis, but requires no special apparatus. The sodium chloride in the early stages of washing acts as a solvent for any calcium phosphate present and, in addition, inhibits undue swelling of the gelatin in the acid liquors.

EXPERIMENTAL RESULTS.

Water absorption under varying conditions was studied by weighing the swollen pieces of gelatin after the attainment of equilibrium with the surrounding fluid, usually after 3 days. The weights of the swollen gelatin are expressed in the final records as a percentage on the dry weight at 100°. The values obtained are not absolute equilibrium figures, since gelatin goes on slowly absorbing water for an indefinite period [Jordan Lloyd, 1920], but they represent the end of the first rapid series of changes due to the influence of the electrolytes on the physical state of the gelatin, the later slow changes being mostly due to hydrolysis. The p_H value of the fluids in equilibrium with the gelatin were all obtained electrometrically. No measurements were made of the possible fixation of sodium chloride by the gelatin.

Three variables influencing water absorption were examined—p_H, temperature (t) and salt content (M). The experimental results are summarised in the curves shown in the figures.

In reviewing the curves shown in the different figures, it should be borne in mind that gelatin is a substance which undergoes a reversible change with change of temperature between two structurally distinct forms known as gelatin A and B [Smith, 1919]. These differ in rotatory power and in solubility, A being nearly insoluble in distilled water, while B has a considerable solubility. In simple gelatin-water systems the whole of the gelatin is present as the A form at temperatures below 15°, and as the B form at temperatures above 35°. The curves shown at 0° and 12° should, assuming for the moment that the presence of the electrolytes does not appreciably affect the proportions of the two forms, indicate systems containing only gelatin A, those at 18° and 24° systems containing mixtures of A and B, although at 18° the proportion of B may be assumed to be very small, while at 24° it will be roughly 50 %. A comparison of the curves in the different figures does actually show very similar behaviour of the systems at 0°, 12° and 18° and a marked contrast at 24°.

The influence of p_H (hydrochloric acid or sodium hydroxide) on the swelling of gelatin at 18° (gelatin A) is shown in Fig. 1. In the absence of inorganic salts the swelling curve has a minimum at p_H 5·0, which may therefore be taken as the isoelectric point, and has maxima in the acid range at p_H 2·6 and in the alkaline range at p_H 9·8. On the alkaline side of the isoelectric

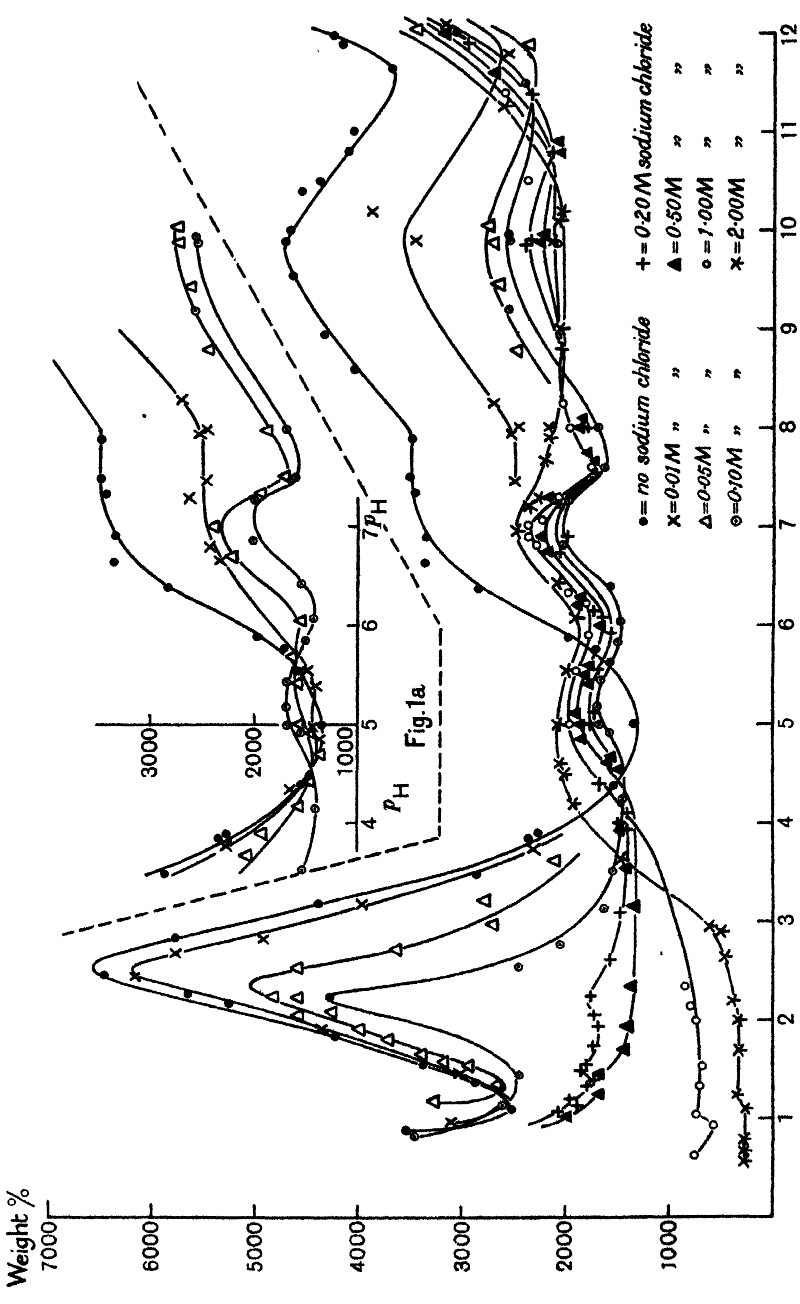

Fig. 1. p_H varying, t constant at 18°, M constant over a series of concentrations from zero to 2·0 M.

point, but close to absolute neutrality (between p_H 7·5 and 7·9), there is an additional broadly defined maximum in the curve of alkaline swelling. At $p_H < 1·1$ or $> 11·6$ the gelatin dissolves in the external fluid.

It will be convenient to consider separately the behaviour of gelatin in the four zones of hydrogen ion concentration centring respectively on p_H 2·6, 9·8, 5·0 and 7·0. These will be designated as the acid, alkaline, isoelectric and neutral zones.

In acid systems the addition of sodium chloride suppresses the swelling due to the acid, and with increasing concentration ($> 0·5\ M$) actually leads to a coagulation of the gelatin (Figs. 1, 7).

The acid swelling zone (p_H 1·5 *to* 3·5).

The swelling of gelatin gels in acid solutions was first shown by Procter [1914] to be an example of Donnan's well-known phenomenon of membrane equilibrium. The combination of the weak base gelatin with part of the acid present to form soluble, ionisable and hydrolysable salts leads to the production of a non-diffusible cation and one or more diffusible anions and hence to an unequal distribution of the diffusible ions across the gel surface with an excess of diffusible ions inside the gel, the presence of which gives rise to an internal osmotic pressure leading to the absorption of water by the gel. Loeb [1922] has also shown that, between certain limits, the repressive action of metallic salts on the osmotic pressures of gelatin solutions with acid reactions is due to their influence on the membrane equilibrium. Loeb equates the internal osmotic pressure of a gelatin-hydrochloric acid-sodium chloride system to $2y + z - 2\sqrt{y\,(y + z)}$, where z is the concentration of the chlorine ion due to the ionisation of the gelatin chloride, and y the sum of the chlorine ions due to the hydrochloric acid and the sodium chloride. Loeb considers that as y increases while z remains constant, in the limit when y is very large compared to z, the expression $(2y + z)/2\sqrt{y\,(y + z)}$ must equal 1, hence $2y + z = 2\sqrt{y\,(y + z)}$ and the internal osmotic pressure falls to zero. Now when y is large and z is small, the expression $(2y + z)/2\sqrt{y\,(y + z)}$ does not differ greatly from the expression $(y + z)/\sqrt{y\,(y + z)}$. Now $y + z$ is the concentration of the chlorine ions in either a gelatin solution or jelly in equilibrium with an external solution in which the concentration of chlorine ions is $y\,(y + z)$, and Loeb has shown that in the case of a gelatin sol separated from its external fluid by a membrane, a direct titration of the chlorine ions in both the inner and the outer solutions [Loeb, 1922, pp. 183–4] shows that at 25° the value of $(y + z)/\sqrt{y\,(y + z)}$ becomes 1·06, *i.e.* approximates to 1, as the salt concentration reaches 0·062 M ($M/16$). Moreover, he shows that if osmotic pressure and the value of the expression $(y + z)/\sqrt{y\,(y + z)}$ be plotted against the logarithm of the salt content, the two curves are parallel (p. 183), both being S curves which ultimately run parallel to the axis of salt concentration. If the effect of the metallic salts on the volume of the jelly is limited, therefore, to an effect on the membrane equilibrium, and if the contribution of the colloidal ions to the internal osmotic pressure is taken as negligible, then it might be anticipated that increasing the concentration of salt up to 0·06 *M* would reduce the jelly to the same volume as in distilled water, after which further increase in salt concentration would have little or no effect. Such is, however, by no means the case. A reference to Figs. 1 and 7 shows that in the acid zone increasing concentration of sodium chloride has an increasing repression on gel volume up to a concentration of 2 *M* and possibly beyond. At 0·5 *M* sodium chloride the swelling in acid solutions is very close to that in distilled water, but at 1·0 and 2·0 *M* the water absorption is far below that in distilled water. The experimental curves showing swelling with varying salt content in acid solutions (Fig. 7) are not of similar form to Loeb's theoretical curve. On the contrary, their general form suggests that they represent a compound function, the sum of two simple functions of *M*, the salt concentration. When $\log M < -0{\cdot}5$ the swelling curve resembles in form the curve of Loeb's equation, but when $\log M > -0{\cdot}5$ the swelling is a negative linear function of $\log M$.

It follows, then, that sodium chloride in acid solutions represses swelling by some additional effect as well as by its influence on the membrane equilibrium. This second effect only becomes patent in comparatively strong solutions (0·5 *M* or greater).

The swelling in the acid zone is strongly influenced by temperature, the volume of the jelly in salt-free systems being apparently an exponential function of *t* until on the verge of solution (Fig. 2). An acid gel at p_H 2 passes completely into solution at about 20°. This is not due to the appearance of gelatin B in the system, since there is no sudden turning of the volume-temperature curve at about 15–18° as there is in the case of the isoelectric and neutral systems. The influence of temperature on the water absorption of acid gelatin is mainly due to its influence on the ionisation of the weak base gelatin and on the hydrolysis of its salts, with the consequent influence on the membrane equilibrium. A comparison of the acid-binding power of gelatin, as given in the figures of Jordan Lloyd and Mayes [1922] at 20° and of Atkin and Douglas [1924] at 25°, shows that between p_H 5 and 2·5 more acid is fixed at the higher temperature. The temperature effect is greater between p_H 5 and 2·5, at which point saturation of the amino-groups of the gelatin is complete, than between p_H 2·5 and 1. The rotatory power of gelatin salts differs from that of free gelatin [Jordan Lloyd, 1922], but the evidence does not suggest that salt formation is accompanied by a transformation of form A of gelatin into form B. The electrically charged gelatin A as well as gelatin B can form a colloidal solution.

The alkaline swelling zone (p_H 8 *to* 11·5).

It is not as easy to make accurate and reproducible experiments on the properties of gelatin in alkaline solutions as it is in acid solutions. All experiments and estimations of p_H, etc. must be carried out in closed vessels to prevent the absorption of carbon dioxide from the atmosphere. There is, in addition, the difficulty that, even at low temperatures, the hydroxyl ion catalyses the hydrolysis of gelatin very much more than the hydrogen ion. Perhaps for this reason the zone of alkaline swelling has not been studied so much as that of acid swelling. On referring to Fig. 1 it can be seen that the curve of alkaline swelling in pure sodium hydroxide solution is not symmetrical with the acid-swelling curve. As the p_H value moves from 5·0 to 7·0 the volume of the gel rises to a steady level; then, with further increase from 7·9 to 9·8, to a true maximum. With further increase in alkalinity the gel volume decreases up to p_H 11·5, at which value at 18° solution of the gel sets in. Loeb [1922, p. 81] shows a curve of similar form at 25°. The p_H-swelling curve may be compared with the curve of combination with base at varying p_H. Atkin and Douglas [1924], working at 25°, show that the combination of base with protein takes place in two stages, the first complete at p_H 7–8 and the second at p_H 11–12. Although on the acid side p_H 2·6 marks both maximum combination with acid and maximum swelling, on the alkaline side maximum swelling occurs at p_H 9·8, a point which does not correspond with either of the two maxima of combination indicated by Atkin and Douglas. Neither of these maxima, however, is sharply defined, and Reiner [1926] has suggested that the influence of the gelatin anion on the activities of the other ions causes errors in the calculated results. It seems at least highly probable that alkaline swelling with a sharp maximum at 9·8 is due to the formation of salts between the carboxylic groups of the gelatin and the sodium hydroxide, and that the series of sodium gelatinates ionises, with the formation of colloidal anions which lead to an unequal distribution of the diffusible ions between jelly and external fluid, with an excess of diffusible ions in the jelly, leading to an internal osmotic pressure and the absorption of water.

The maximum of alkaline swelling at p_H 9·8 is in all ways the counterpart of the acid peak of swelling at 2·6. The sharpness of the peak, the relative positions of both ascending and descending branches, and the repression of the alkaline swelling by the addition of sodium chloride (Figs. 1 and 7), all suggest that the two systems are similar. The behaviour of sodium gelatinate in the presence of sodium chloride is, however, apparently simpler than that of gelatin hydrochloride. From the curves shown in Figs. 1 and 7 it can be seen that sodium chloride solutions have no coagulating action on alkaline gelatin and apparently the effect of adding this salt in the alkaline zone is limited to its effect on the membrane equilibrium. The repressive action of the salt on swelling seems to reach its full effect in 1·0 *M* solutions. The curve of swelling-log *M* at p_H 10 (Fig. 7) turns and runs parallel to the axis of log M in accordance with the theoretical expectations implied in Loeb's equation. The volume of the jelly, however, is never brought back to the volume in distilled water, a fact which suggests that the osmotic pressure of the gelatin ions is not negligible.

The effect of temperature on swelling in the alkaline zone is similar to that in the acid zone, and therefore mainly due to the influence of temperature on the ionisation and hydrolysis constants of the gelatin. Fig. 2 shows the effect of temperature on swelling at p_H 10·5. Swelling increases very rapidly with temperature and is, as with acid jellies, an exponential function of t until the verge of solution. There is no sign of an abrupt change in the form of the curve between 15° and 18° and the jelly passes into solution at about 20°. The rotatory power of the salts of gelatin with bases differs from that of the free gelatin [Jordan Lloyd, 1922], but the evidence does not suggest that the formation of sodium gelatinates is accompanied by a transformation of the A form of gelatin into the B form. The negatively charged particles of gelatin A form a colloidal solution.

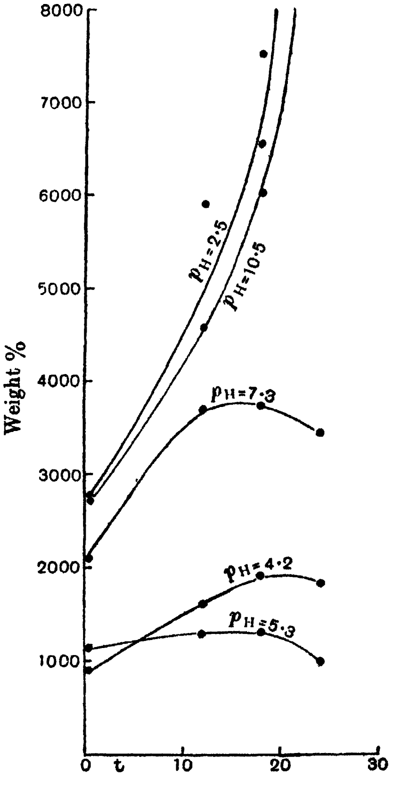

Fig. 2. t varying, M constant at zero, p_H constant at 2·5, 4·2, 5·3, 7·3 and 10·5.

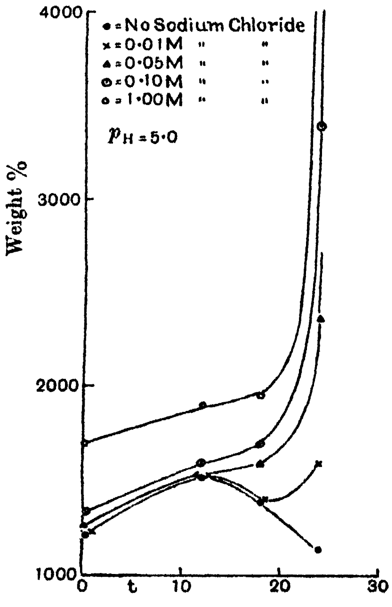

Fig. 3. t varying, p_H constant at 5·0, M constant at five values varying from zero to 1·0 M.

The isoelectric zone (p_H 4·6 *to* 6·0).

The position of minimum swelling in a system free from sodium chloride lies at p_H 4·9 to 5·0, which may be taken as the isoelectric point of the pure gelatin. The presence of sodium chloride in isoelectric or nearly isoelectric solutions causes absorption of water by gelatin A, the amount absorbed at constant temperature being (except in very dilute solutions) proportional to the logarithm of the concentration (Fig. 5, curves at 0°, 12° and 18°). The effect of sodium chloride on water absorption at 24°, when gelatin B may also be assumed to be present, is not directly proportional to log M, but to $e^{\log M}$ (Fig. 5). The p_H of the solution has a marked effect on the influence of the chloride, swelling induced by the action of the salt being greatest at the isoelectric point where the positive and negative gelatin ions are either absent or at a minimum, and increasingly less with increasing concentration of either hydrogen or hydroxyl ions, *i.e.* with increasing concentration of the gelatin ions (Fig. 1). The p_H range over which the swelling effects of sodium chloride are shown is very narrow in dilute solutions, but becomes increasingly wider with increasing salt concentration—at 0·01 M sodium chloride it extends from p_H 4·4 to 5·6 and at 2 M from p_H 3·5 to 6·0 (Fig. 1). The salt influence is evidently limited to the electrically neutral gelatin, and the swelling of gelatin under these conditions is due to an entirely different mechanism from swelling under the influence of the hydrogen or hydroxyl ion.

The effect of temperature on swelling in the isoelectric zone differs strongly from that in the acid and alkaline zones. Below 15° there is a gradual increase of volume with temperature in the salt-free system and at all concentrations of sodium chloride up to 2 M. The influence of the temperature is therefore directly due to action on particles of gelatin A, present in the jelly in the gel form, probably leading to a weakening of the cohesive forces of the rigid structure of the jelly above 15°. The effect of temperature in the absence of salt or at low dilutions (0·01 M) differs from its effect at higher concentrations in an important and striking manner; in the two former cases there is, with further rise of temperature, a decrease in swelling, in the latter a rapid increase (Figs. 2 and 3). This leads to the impression that gelatin B is, in the absence of salt, less hydrated than gelatin A, but is more readily dispersed by sodium chloride. This has already been observed in the smooth but steep rise of the curve showing the influence of salt concentration on swelling at 24° (Fig. 5).

Sodium chloride increases the hydration of electrically neutral gelatin A, but does not disperse it to form a sol. It has a very marked dispersing action on gelatin B.

The zone of neutrality (p_H 6 *to* 8).

Most proteins are isoelectric in solutions which are slightly acid, hence solutions which are neutral, assuming for the moment that neutrality may be defined with sufficient accuracy as any p_H value between 6 and 8, must contain small quantities of base, both free and combined with the protein.

In a system of gelatin jelly and sodium hydroxide free from diffusible salts, an increase of p_H from 5 towards the more alkaline regions leads at first to a fixation of the base by the protein and to a swelling which increases with increasing p_H. At 18° the swelling reaches a steady level between p_H 7·4 and 7·9. At 25°, and at about the same reaction, the combination of protein and base also reaches a steady level. The swelling can therefore be considered as due to the formation of partially ionisable salts. Evidence of the extent of the ionisation is, however, somewhat conflicting. Reiner has pointed out [1926] that immediately on the alkaline side of the isoelectric point there is a zone where there is no migration of protein in an electric field [see also Svedberg, 1924; Cohn, 1925]; hence, therefore, a zone in which there is no ionisation of the protein salt. Experiments here recorded on the influence of salts in the neutral zone (Fig. 1 *a*) suggest that part of the sodium gelatinates is present in the ionised form, since the first effect of adding sodium chloride in the neutral zone is exactly the same as in the acid and alkaline zones, *i.e.* there is a repression of swelling which can be accounted for in the usual manner as due to a readjustment of a membrane equilibrium. In 0·01 *M* solutions of sodium chloride this is the only effect produced by the salt. At 0·05 and 0·10 *M*, although there is increased repression with the increased salt concentration, the curve of swelling has taken on a different form, strongly suggesting that, although the salt is repressing swelling by one chemical mechanism, it is simultaneously promoting it at about p_H 7 by another. At concentrations from 0·10 to 2·0 *M* the effect of the salt is exactly the same as in the isoelectric zone, *i.e.* at 0°, 12° and 18° the swelling of the jelly increases in proportion to the logarithm of the concentration of the salt (Figs. 1, 6 and 7).

The volumes attained at 18° under the influence of salt are rather greater at p_H 7 than at p_H 5, and, although precise figures are not available, it has been ascertained that the melting-point of the jelly is lower at p_H 7. It is an interesting point that, although jellies can swell to about 7000 % of their dry weight under the action of acid or alkali without going into solution, the presence of sodium chloride leads to the conversion of gel into sol if the swelling approaches 3000 %. In the former case only the gelatin ions pass into the sol state, in the latter the electrically neutral gelatin of the gel framework is dispersed.

The influence of temperature on swelling in the neutral zone (Fig. 2, curve at p_H 7·3) shows an interesting compromise between its influence at p_H 5·3 and at p_H 10·5. In the absence of salt and at the lower temperatures, swelling increases nearly as steeply with temperature as in the alkaline zone, at the higher temperature ($t = 18°$) further rise of temperature causes a repression of swelling as in the isoelectric zone. The influence of temperature in the salt-containing systems (Fig. 4) shows again the mixture of two effects. At the lower salt concentrations swelling increases with temperature up to about 20° at 0·01 *M* concentration and to 22° at 0·05, after which point further rise of temperature leads to loss of volume. With increasing salt concentration the optimum temperature of swelling rises, but after 0·1 *M* has been reached the curves take on a form resembling those obtained at p_H 5 (Fig. 3).

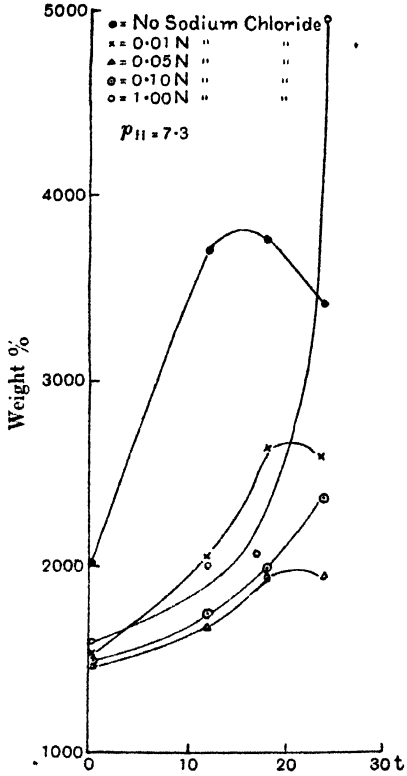

Fig. 4. *t* varying, p_H constant at 7·3, *M* constant at five values varying from zero to 1·0 *M*;

DISCUSSION.

The water content of a gelatin jelly is determined by the balance of a number of factors. The jelly consists of a solid framework of gelatin in the gel form and an interstitial fluid containing one of the several possible sol forms. Unless both sol and gel forms are present the jelly is unstable [Jordan Lloyd, 1920]. Only gelatin A can assume the gel form, but it seems likely that not only the free ampholyte A but also the unionised salts A.HCl and NaA and possibly the hermaphrodite ions $^+A^-$ postulated by Pauli can all take a place in the framework of the jelly. The characteristic of the gel forms of gelatin seems to be the absence of an electric potential. The sol forms of gelatin may be either electrically charged or electrically neutral; in the former case the sol may be formed by free gelatin ions, A^+ and A^-, in the latter by a dispersion of gelatin A through the action of salts, or by gelatin B. The water content of the jelly is fixed by a balance between osmotic forces in the sol and the elastic force in the gel framework, the latter also containing water present as a solid solution. Reactions which increase the osmotic forces in the interstitial fluid or those which weaken the elastic forces of the framework lead to swelling; conversely, those which decrease the internal osmotic pressure or increase the elasticity lead to shrinking.

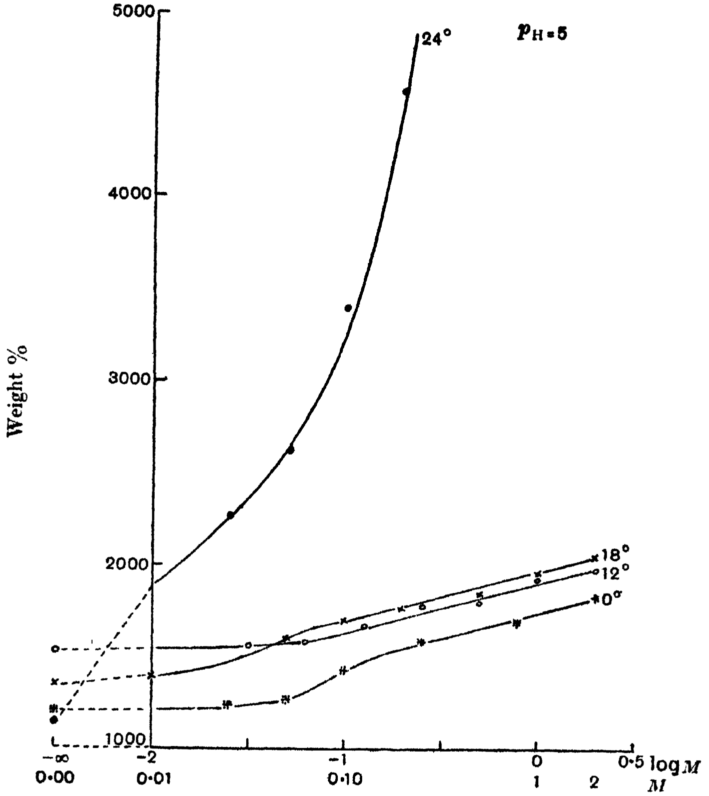

Fig. 5. M varying, p_H constant at 5·0, t constant at 0°, 12°, 18°, 24°.

The formation of ionisable salts between gelatin and hydrochloric acid or between gelatin and sodium hydroxide leads to the appearance in the jelly of colloidal ions with a consequent unequal distribution of diffusible ions across the membrane. The internal osmotic pressure which controls the volume of the jelly is due mainly to the excess in the jelly of the diffusible ions of the gelatin salt, though it can be seen from the results obtained in the alkaline zone that the colloidal ions must contribute some part to the whole. The first effect of the addition of sodium chloride to a system where gelatin hydrochlorides or sodium gelatinates are present in the jelly, is a suppression of swelling which can be accounted for by the readjustment of the membrane equilibrium. This appears to be the only effect of the salts in the alkaline zone. In the acid zone the sodium chloride, after reaching a concentration of 0·5 M, dehydrates and coagulates the gelatin. In saturated sodium chloride solutions the precipitation in the acid zone is complete [Moeller, 1921]. It is tentatively suggested that the excess of sodium chloride dehydrates A^+, the positive gelatin ion, through the greater attraction of its own ion for the dipoles of water. Dokan [1924] has shown that with colloidal carbohydrates strong salt solutions repress swelling apparently by withdrawing the water from the colloid for the hydration of their own ions. The gelatin cation, which other evidence suggests is heavily hydrated, appears to part fairly easily with its water and is therefore flocculated in concentrated salt solutions. It is especially to be noticed that at a concentration of salt in which neutral or isoelectric gelatin may be assumed to be adsorbing both the positive and negative salt ions with their associated water, thus becoming hydrated, the gelatin cation is giving up its water to these same ions free in the solution. Presumably the gelatin cation does not adsorb the ions of the salt so freely as the uncharged gelatin.

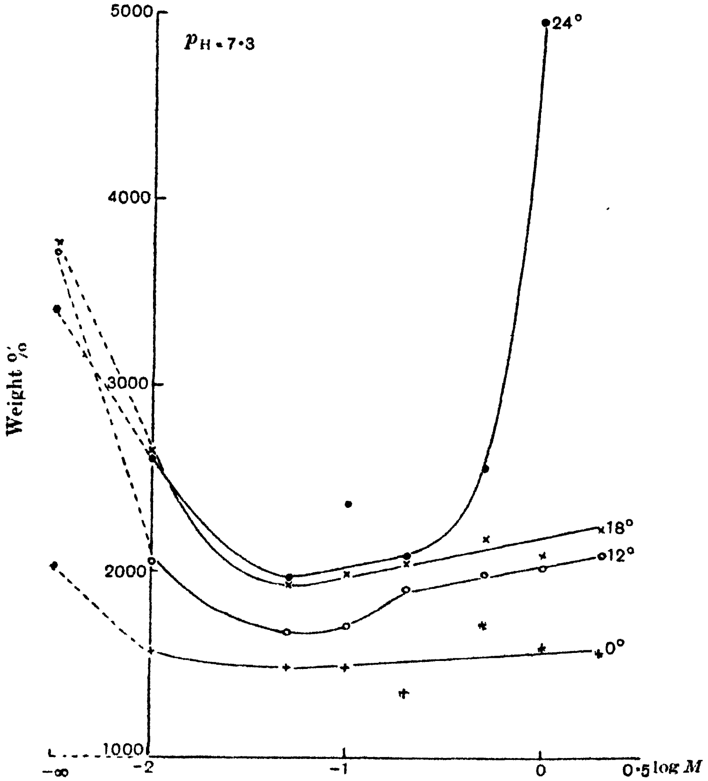

Fig. 6. M varying, p_H constant at 7·3, t constant at 0°, 12°, 18°, 24°.

The formation of gelatin salts in a jelly causes swelling by increasing the osmotic forces in the interstitial fluid and simultaneously weakens the framework and the elastic recoil. The effect of change of temperature under these conditions is that swelling increases with rise of temperature. This effect seems mainly due to the influence of the temperature on the ionisation and hydrolysis constants of the gelatin. With rising temperature the hydrolysis of the gelatin salts (sol forms) and regeneration of uncombined gelatin (gel form) will be diminished. Hence, rising temperature will weaken the elastic forces of the framework and at the same time it will increase osmotic pressures through increasing the kinetic energy of both colloidal and diffusible ions in the jelly.

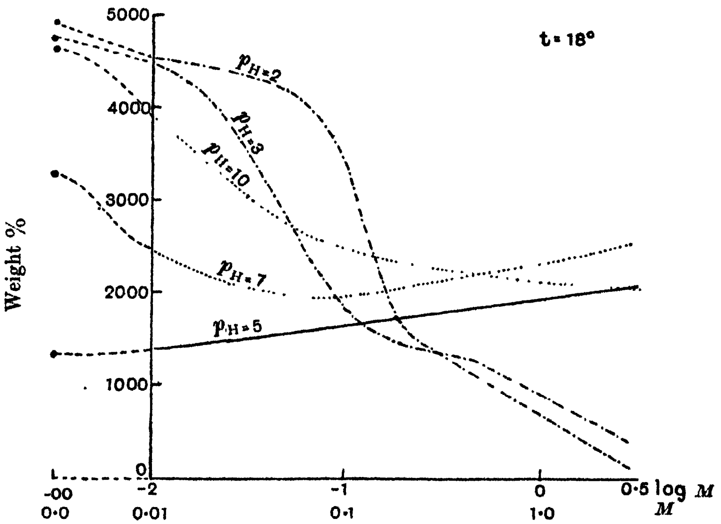

Fig. 7. M varying, t constant at 18°, p_H constant at 2, 3, 5, 7 and 10.

At the isoelectric point the jelly, in equilibrium with water, can be made to swell by a different mechanism from the formation of gelatin salts. At p_H 5 a gelatin jelly free from mineral salts is unstable and the framework collapses under its own forces [Jordan Lloyd, 1920]. With the addition of sodium chloride the conditions for stabilising the jelly appear. The adsorption of both positive and negative ions of the salt by the gelatin, with the resulting hydration of the latter, weakens the elastic forces of the framework. Some of the gelatin will go into the form of an uncharged sol exerting a slight osmotic pressure. Eventually the jelly will be completely dispersed by the salt solution.

Dokan [1924], who has investigated a similar effect of salts on the colloidal carbohydrates, ascribes the action of the salt to the adsorption on to the surface of the electrically neutral colloidal particles of both the positive and negative ions of the salts, each ion carrying into the system its surrounding shell of water. On this theory the swelling curve should resemble the adsorption curve and be a logarithmic function of the salt concentration, a relation which has been shown to be true for systems consisting mainly of gelatin A.

Temperature has an interesting effect here. A rise of temperature from 0° to 15°–18° slightly weakens the elastic forces of the jelly and the volume increases slightly. At 15°–18° gelatin B, the sol form, begins to appear in the system. Sodium chloride has a strongly dispersing action on gelatin B and salt solutions rapidly dissolve jellies with rise of temperature between 18° and 24°. When no sodium chloride is present, however, rise of temperature over the region 18° to 24° leads to loss of volume by the jelly. Gelatin B, the sol form of uncombined gelatin, is ordinarily more soluble in water than gelatin A, but the latter appears to have a greater capacity of dissolving water in itself to form a solid solution. Further evidence of the behaviour of gelatin in salt-free systems at higher temperatures is needed here.

From the biological point of view the most interesting region studied is the neutral zone, since within this lie the conditions of p_H and salt content generally found in the living cell. The theory of the formation of ionisable sodium gelatinates with a consequent internal osmotic pressure and of the influence of sodium chloride on these, explains the volume relations up to a salt concentration of 0·01 *M*. As the concentration of sodium chloride reaches the order of value found in the living cell (approximately 0·1 *M*) the system is found to have undergone a considerable change. The volume now attained by the jelly, both with change of salt concentration and with temperature, is controlled by the same mechanism as at the isoelectric point, or, in other words, as the salt concentration passes a critical value the gelatin becomes electrically neutral and the jelly volume is now due to a balance of osmotic forces derived from an uncharged sol and the elastic forces of a framework weakened by the adsorption of the hydrated ions of the sodium chloride. The effect of sodium chloride at p_H 7 is greater than at p_H 5. Pauli [1926] considers that at the isoelectric point solutions of proteins may contain the protein in four different forms: the charged ions A^+ and A^-, the uncharged pseudo-ampholyte A and the hermaphrodite ion of the internal salt form $^+A^-$. The first additions of acid are said to increase the concentrations of $^+A^-$. It must remain for the present an interesting speculation whether the first additions of alkali have a similar but possibly even greater effect. Other salts are now under investigation in order to provide further evidence for or against the hypotheses advanced in the paper.

SUMMARY.

1. Sodium chloride, in the presence of hydrochloric acid and up to a concentration of 0·01 M, suppresses swelling of gelatin due to the acid; at greater concentrations it causes a coagulation of the acid gelatin. The maximum of acid swelling is at p_H 2·6.

2. Sodium chloride in the presence of sodium hydroxide suppresses the swelling of gelatin due to the alkali. Even at high concentrations (2 M) the salt has no further action. Maximum alkaline swelling is at p_H 9·8.

3. The swelling of gelatin due to acid or alkali increases as an exponential function of the temperature.

4. The point of minimum swelling of ash-free gelatin (isoelectric point) is at p_H 5·0. At this point sodium chloride promotes water absorption, swelling being proportional to the logarithm of the concentration of the salt.

5. At p_H 5, in the absence of salts, swelling increases slowly with rising temperature up to 15° or 18°, after which, with further rise, there is a decrease of swelling.

6. At p_H 5 the presence of sodium chloride increases swelling slowly with temperature up to about 18°, after which there is very rapid increase ending at about 20° in complete solution of the gelatin. The greater the concentration of sodium chloride present, the lower the temperature at which solution occurs.

7. At p_H 7, in the absence of salts, gelatin shows some swelling due to the alkali present. The addition of sodium chloride up to 0·01 M causes a suppression of swelling; at greater concentrations the salt, as at p_H 5·0, induces a swelling which is in logarithmic ratio to its concentration.

8. At p_H 7, in salt solutions stronger than 0·01 M, the effect of temperature on swelling resembles its effect at p_H 5. When the concentration of sodium chloride is less than 0·01 M, or in the absence of salts, the effect of temperature on swelling at p_H 7 is intermediate between its effects in the alkaline and in the isoelectric zones.

9. When gelatin is in solution as electrically charged particles the effect of adding sodium chloride is mainly electrostatic; when present in electrically neutral forms, the salt acts by adsorption and hydration.

10. The theoretical aspects of swelling are discussed.

In conclusion we should like to thank the Council of the British Leather Manufacturers' Research Association for permission to publish this work and the Director (Dr R. H. Pickard, F.R.S.) for the interest and criticism which he has given us during its progress.

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CLXXIX. NOTE ON THE ATTEMPTED ACTIVATION OF TYROSINE BY ULTRA-VIOLET IRRADIATION.

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IN the course of a recent paper on the ultra-violet irradiation of rations Waltner [1927] has claimed that pure tyrosine may be rendered antirachitic by such treatment. Although this statement finds no support in the theory of specific sterol activation now generally accepted, the importance of tyrosine in melanin formation on the one hand, and the relation of protective pigmentation to irradiation on the other, led us to put the matter to independent test. As a result we are unable to confirm the view that tyrosine may be rendered antirachitic.

EXPERIMENTAL.

A sample of tyrosine, to ensure freedom from impurities of a fatty nature, was extracted with ether for 48 hours in a Soxhlet apparatus. One portion was then exposed in a thin layer before a mercury arc lamp for a period of 20 minutes at a distance of 50 cm., the remainder was retained for use in the negative control diet. For use in the positive control diet a sample of ergosterol supplied by the British Drug Houses was exposed at the same time as the tyrosine to the radiations of the same lamp.

In order to eliminate the possibility of fortuitous healing through fall of food intake, etc., it was decided that the curative method, employed by Waltner, should not be adopted, but that the materials should be tested by their efficiency in preventing rickets when introduced into the diet from the outset. Twelve young albino rats were divided into three groups. Two rats (Group I) received the McCollum diet 3143 with the daily supplement of 0·1 mg. of irradiated ergosterol suspended in liquid paraffin; five rats (Group II) received diet 3143 with the admixture of 0·5 % of irradiated tyrosine; the remaining five received the same diet containing a similar amount of tyrosine which had not been irradiated. After 45 days the rats were killed and their bones examined, with the results shown in the table.

It will be seen that whereas almost normal bones were observed in the case of the two rats which had received irradiated ergosterol, no distinction in the severity of rickets could be drawn between the remaining two groups, symptoms in the case of rats which had received irradiated tyrosine being as gross as in the case of rats which had received the material without irradiation. Therefore, while such results do not rule out the possibility that irradiated tyrosine may have some slight healing effect on rickets in its early stages, it is obvious that its continued administration cannot prevent or moderate the progress of rickets in the case of the rat.

Table I.

	Weight (g.)	State of ribs	Diagnosis
Group I (irradiated ergosterol).			
♂	42–92	Normal for 3143 diet	Normal
♀	39–74	Normal for 3143 diet	Normal
Group II (irradiated tyrosine).			
♀	47–92	Costochondral junctions blurred. Three ribs fractured. Marked angulation	Severe rickets
♀	49–72	Junctions much enlarged with cupping, lines blurred. Four ribs fractured. Some angulation	Severe rickets
♂	39–90	Junctions much enlarged, lines fairly distinct. Two ribs fractured. Marked angulation	Severe rickets
♂	45–82	Junctions enlarged with lines slightly blurred. Four ribs fractured. Slight angulation	Severe rickets
♂	49–76	Junctions almost normal, but two ribs fractured. No angulation	Slight rickets
Group III (non-irradiated tyrosine).			
♀	40–71	Junctions enlarged and cupped, lines blurred. Four ribs fractured with partial collapse of thorax on left side	Very severe rickets
♂	44–61	Junction lines fairly distinct. Eleven ribs fractured. Severe angulation	Very severe rickets
♂	46–109	Junction lines fairly distinct. One rib fractured. Angulation on left side	Moderate rickets
♂	49–116	Junction lines rather blurred. Very slight angulation	Slight rickets
♀	52–102	Junctions slightly enlarged and lines rather blurred. No angulation	Very slight rickets

SUMMARY.

Irradiated tyrosine admixed at the rate of 0·5 % in the McCollum diet 3143 is ineffective in moderating the course of rickets in rats.

Our thanks are due to Sir F. G. Hopkins for his support and criticism and to Prof. Drummond for the use of a mercury arc lamp.

REFERENCE.

Waltner (1927). *Magyar Orvosi Arch.* **28**, 19.

CLXXX. THE ACTION OF TYROSINASE ON PHENOLS.

WITH SOME OBSERVATIONS ON THE CLASSIFICATION OF OXIDASES.

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It has been known for many years that tyrosinase will bring about the oxidation of certain phenols, and the reaction with *p*-cresol has been used by Chodat [1912] as an effective means of identifying the enzyme. In the presence of amino-acids the reaction with *p*-cresol produces a series of brilliant colour changes, and in addition deamination of the amino-acid takes place. It was shown by Happold and Raper [1925], and almost at the same time by Robinson and McCance [1925], that the deamination was not due, as Chodat thought, to a direct action of tyrosinase on the amino-acid, but to the intervention of the phenol in the reaction. Happold and Raper [1925] brought forward evidence that *ortho*-quinones were first produced from those phenols which were attacked by tyrosinase, and that the deamination produced in the system, tyrosinase-phenol-amino-acid, was due to action of the *o*-quinone thus produced on the amino-acid.

It was shown at that time that *o*-benzoquinone would bring about the deamination of alanine and glycine under the same conditions as to reaction of the medium and temperature, which prevailed in the enzyme action. This evidence has been extended in the present paper by showing that *o*-homoquinone behaves in a like manner towards glycine. Additional indirect evidence of the formation of *o*-quinones from phenols by the action of tyrosinase has been put forward by Raper [1927] in explaining the action of this enzyme on tyrosine. In the series of changes which take place in this reaction, and which result finally in the production of melanin, it seems very probable that the 3.4-quinone of phenylalanine is one of the intermediate products. It has been shown by Onslow and Robinson [1925] that the system *p*-cresol-tyrosinase, and by Szent-Györgyi [1925] that *o*-quinone, turns tincture of guaiacum blue, and since the product of the action of tyrosinase on catechol and certain other phenols will turn guaiacum blue it has been suggested by the latter that *o*-quinones are produced by the action of the enzyme on these phenols.

Since all this evidence is indirect it seemed desirable to establish it more securely, if possible, by the isolation of compounds of *o*-quinone from the reaction system. *o*-Benzoquinone is a very unstable substance and can be prepared only in dry ether, and *o*-homoquinone is not much more stable. It seemed likely therefore that these substances, if produced by the action of tyrosinase on phenol or *p*-cresol, would have only a transient existence, and that to isolate them some substance must be used to withdraw them from the sphere of reaction. Aniline, upon which tyrosinase has no action, was found to be suitable since it forms with quinones insoluble anilinoquinones. Benzenesulphinic acid, which has been shown by Hinsberg and Himmelschein [1896] to combine with *o*-benzoquinone, was also tried instead of aniline with the hope of being able to isolate such a compound from the products of the action of tyrosinase on phenol and on catechol. It was however successful only with catechol.

The anilinoquinones produced, as described above, by the action of tyrosinase on phenol, catechol, *p*-cresol, *m*-cresol, and homocatechol in presence of aniline, have been isolated, and identified by synthesis. It has also been shown by the same method that peroxidase and hydrogen peroxide acting on catechol and on homocatechol produce *o*-benzoquinone and *o*-homoquinone respectively. It seems, therefore, to be clear that the first stages in the action of tyrosinase on those monohydric phenols on which it acts are the introduction of a second hydroxyl group in the *o*-position to the first, thus producing a catechol derivative, and then oxidation of this to the corresponding *o*-quinone. Peroxidase and hydrogen peroxide also bring about the latter reaction.

Not all phenolic substances which are attacked by tyrosinase give rise to anilinoquinones when the enzyme acts on them in the presence of aniline, but *m*- and *p*-methoxyhydroxybenzene do so. Tyrosine and pyrogallol are examples of substances which, though oxidised by the enzyme, do not yield anilino-compounds under these conditions. There is good evidence, however, that in both these instances *o*-quinones are produced, but, owing to further reactions which these undergo, *i.e.* the formation of indole derivatives from tyrosine [Raper, 1927], and of purpurogallin from pyrogallol [Willstätter and Heiss, 1923], combination with aniline does not take place.

By means of the microrespirometer, some indication has been obtained of the quantitative relations which hold between the amounts of oxygen taken up and of phenols oxidised. The results show that in those instances in which it has been proved that *o*-quinones are formed, the oxygen taken up is greater than the amount required for the formation of the *o*-quinone. The enzyme preparation used in these experiments contained catalase, so that the excess oxygen uptake over and above that which is required for the formation of the *o*-quinone, cannot be accounted for simply by the formation of hydrogen peroxide as represented, for example, in the reaction

$$C_6H_4(OH)_2 + O_2 = C_6H_4O_2 + H_2O_2,$$

in which oxygen is acting as a hydrogen acceptor. It would appear that the *o*-quinones take part in some further reaction or reactions which involve oxidation. The nature of these has not yet been discovered.

The results described in this paper together with those obtained by other workers in this field during the past few years suggest that the hypothesis as to the nature of the "direct oxidases" which was put forward by Bach and Chodat [1903] requires revision. The "direct oxidases" are usually detected by the fact that they will turn guaiacum tincture blue without the addition of hydrogen peroxide or other peroxides. This distinguishes them from peroxidases or "indirect oxidases" which only give this reaction when peroxides are added. Bach and Chodat believed that peroxidases were an essential component of the "direct oxidases," and that the reason why the latter turned guaiacum blue directly was that a second component was present which in presence of air produced a peroxide. Thinking that this second component was enzyme-like in its action they named it oxygenase. But we now know that peroxidase is not an essential component of the "direct oxidases." It has been shown by Szent-Györgyi [1925] that *o*-quinone will turn guaiacum blue, and in the present paper proof is presented that *o*-quinones are produced by the action of tyrosinase on certain phenols, some of which, *e.g.* catechol and some of its derivatives, are known to be present in plants. Furthermore, thermostable catalysts known as polyphenolases have been prepared from plant products by Euler and Bolin [1908] and Wieland and Fischer [1926], which oxidise dihydric phenols to quinones with the formation of hydrogen peroxide. In the presence of catechol or some catechol derivative and even in the absence of peroxidase, these catalysts would cause the direct blueing of guaiacum because of the production of *o*-quinones. Since these systems give the guaiacum reaction it has therefore ceased to be an accurate guide to the presence of a "direct oxidase" of the kind postulated by Bach and Chodat. Altogether four different oxidation systems have now been shown to give the "direct oxidase" reaction.

(1) Peroxidase + an autoxidisable substance which produces hydrogen peroxide or an organic peroxide in air. Gallagher [1923] has found a system of this type in the potato, the autoxidisable substance being a lipin or a mixture of lipins. It seems likely that many unsaturated substances, *e.g.* terpenes or unsaturated fats or fatty acids would behave likewise. Bertrand's laccase probably comes into this group.

(2) Peroxidase + an oxidoreductase and its substrate. An example of this system is that investigated by Thurlow [1925] in her studies of the xanthine oxidase of milk, which, when acting on xanthine or hypoxanthine in presence of air, gives rise to the production of hydrogen peroxide.

(3) Peroxidase + a polyphenolase with a polyphenol as substrate. Euler and Bolin [1908] have found a system of this type in *Medicago sativa.* Their polyphenolase was thermostable and was apparently a mixture of calcium salts of organic acids. Its action could be imitated by a mixture of calcium glycollate and manganese acetate. If the polyphenolic substrate is a catechol derivative, the peroxidase is not necessary to give the "direct oxidase" reaction, since the *o*-quinone produced by the action of the polyphenolase would blue guaiacum by itself. In this group would come also the thermostable polyphenolase isolated by Wieland and Fischer [1926] from *Lactarius*. This was different in composition from that obtained by Euler and Bolin but exerted the same type of action.

(4) Tyrosinase + one of its substrates (tyrosine, 3 . 4-dihydroxyphenylalanine and pyrogallol excepted). This is the system described in the present paper.

It is clear from the composition of these various systems that the term "direct oxidase" is a misnomer if, by it, we wish to denote only one particular oxidising enzyme or oxidation system, and it would be better to abolish it. The "direct oxidase" reaction is essentially a test for the presence of those oxidases and thermostable oxidation catalysts which will bring about oxidation only in the presence of oxygen or peroxides, and the authors suggest that they be called *aerobic oxidases.* These agents are different from the oxidoreductases, which will act in the absence of oxygen provided a hydrogen acceptor is present, and which may be called *anaerobic oxidases.* The oxidoreductases are believed to act by activating hydrogen, but whether this is also true of the aerobic oxidases is at present uncertain. Wieland and Fischer [1926] suggest that the polyphenolase which they have investigated acts by activating hydrogen and not by activating oxygen in the sense that certain metallic ions are supposed to do. Their suggestion of the mechanism is that the catalyst, polyphenol and molecular oxygen unite to form an unstable compound which then breaks down, giving the quinone and hydrogen peroxide. Whether tyrosinase acts in this way is not yet decided in view of the lack of experimental evidence; but Willstätter and Weber [1926] explain the action of peroxidase by the formation of a compound between the enzyme and hydrogen peroxide in which the oxygen is more active than in hydrogen peroxide itself. Although it is clear therefore that the aerobic oxidases act only in the presence of oxygen or a peroxide, it is by no means decided whether they activate oxygen or hydrogen.

In addition to the hypothesis regarding the constitution of the "direct oxidases" which was put forward by Bach and Chodat, an alternative scheme has been suggested by Onslow [1920]. It is that the oxygenase of Bach and Chodat is really a mixture of an enzyme proper—to which Onslow applies the name oxygenase—and a catechol derivative. This view was based essentially on the separation from plants that give the "direct oxidase" reaction, of an alcohol-insoluble enzyme and an alcohol-soluble substance which gave the typical green colour with ferric chloride which is characteristic of catechol derivatives. Neither of these alone gave a definite "direct oxidase" reaction, but when combined gave it very markedly. The results described in the present paper, however, show that tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative. The polyphenolases of Euler and Bolin and of Wieland and Fischer would also give Onslow's oxygenase reaction. There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow.

Nor does this term convey a precise meaning in the sense in which Bach and Chodat first employed it, because, as shown above, it may represent a different component in each one of the four known "direct oxidase" systems.

It is suggested, therefore, that in view of the present state of our knowledge, oxidases should be provisionally divided into an aerobic and an anaerobic group. Those of the former act only in the presence of oxygen or a peroxide, and comprise peroxidase, tyrosinase, and the thermostable catalysts known as polyphenolases. Those of the latter include the known oxidoreductases, and the thermostable catalysts which act in conjunction with glutathione. They will act anaerobically provided a suitable hydrogen acceptor is present.

EXPERIMENTAL.

The enzyme used in this investigation was prepared from mealworms as described by Raper [1926]. In experiments in which enzyme was used, chloroform (unless otherwise stated) was added as antiseptic. The p_H of solutions was adjusted by means of phosphate buffer.

*The isolation of anilino-*o*-quinones.*

o-Quinones being highly reactive and unstable substances, it was not expected that it would be possible to isolate them as such from solutions of phenols which had been submitted to the action of tyrosinase. Attempts were therefore made to remove them from the sphere of reaction by introducing aniline into the system and isolating them as anilinoquinones. It was found that by the action of tyrosinase on phenol in presence of aniline a bright red precipitate was obtained, which crystallised from ether as bright red needles having the characteristics of an anilinoquinone. Tests showed that a maximum yield of this deposit was obtained at p_H 6·5–7·0. The experiments were therefore carried out at p_H about 6·7. The action of tyrosinase in presence of aniline was investigated first of all on phenol and on catechol, then on the cresols and on homocatechol.

Controls. A preliminary control showed that aniline in phosphate buffer at p_H 6·7 is unchanged by tyrosinase. Phenol in buffered aniline water remained unchanged. Catechol became slowly pink, and after some time gave a reddish brown precipitate, this being due presumably to autoxidation to *o*-benzoquinone which then reacts with the aniline. In presence of hydrogen peroxide the oxidation was more rapid. *p*-Cresol and *m*-cresol in buffered aniline water remained unchanged. Homocatechol underwent fairly rapid oxidation, a red substance and some tar being produced by reaction with aniline. The oxidation was hastened by hydrogen peroxide, but in no case was the product obtained nearly so rapidly or definitely as when enzyme was present.

Buffered solutions of phenol and catechol became darkened by the action of tyrosinase, and catechol was also darkened by the action of peroxidase and hydrogen peroxide. Buffered solutions of *p*-cresol, *m*-cresol, and homocatechol by the action of tyrosinase, and of homocatechol by the action of peroxidase and hydrogen peroxide, became orange-yellow, orange-brown, or reddish brown.

Action of tyrosinase on phenol in presence of aniline.

6 g. phenol and 12 cc. aniline were dissolved in about 1·5 l. of buffer solution, and 120 cc. tyrosinase solution were added. The liquid was placed in two 2 l. flasks, and gently aerated with air drawn through a wash-bottle containing chloroform and water. In a few minutes a red colour developed, the liquid clouded, and later red crystalline needles separated out. After two days the precipitate was filtered off on fluted filters, washed with 1 % HCl to remove excess aniline, then with water, and dried *in vacuo*. In cases where the filtrate was still reddish coloured, a further yield was obtained by acidification with 1 % HCl, or precipitation with $CaCl_2$ and 1 % NH_4OH, or by extraction with ether and evaporation of the ether. The total dried substance was extracted in a Soxhlet apparatus with acetone or light petroleum. The latter was slower in its solvent action. On evaporation of the solution bright red needles separated out. The product was purified by recrystallisation from acetone or light petroleum, or alternatively, by solution in ether and extraction with 0·02 *N* NaOH, the aqueous liquid being acidified with 2 % HCl, and the red precipitate filtered off and crystallised from acetone. Yield, 4·5 g. Properties: bright red needles; M.P. 193°; soluble in organic solvents; soluble in concentrated sulphuric acid, giving an olive green colour, becoming red on dilution by reprecipitation; soluble in alkali and alkali carbonate, giving a purplish red solution, reprecipitated by acid.

Analysis. Found: C, 74·58 %; H, 5·08 %; N, 9·57 %.
Calculated for dianilino-*o*-benzoquinone, $C_{18}H_{14}O_2N_2$:
C, 74·48 %; H, 4·83 %; N, 9·66 %.

Action of tyrosinase on catechol in presence of aniline.

2 g. catechol, 4 cc. aniline, and 40 cc. tyrosinase solution were made up to nearly a litre in buffered solution, and aerated. Most of the precipitate was obtained the first day. The product was treated in the same way as that

obtained from phenol. Yield, 1·75 g. Properties: identical with those of the product obtained from phenol; M.P. 193°. Mixed M.P. with the product obtained from phenol, 193°.

Analysis. Found: N, 9·61 %.
Calculated for $C_{18}H_{14}O_2N_2$: N, 9·66 %.

Action of peroxidase and hydrogen peroxide on catechol in presence of aniline.

As was expected, catechol is oxidised by peroxidase and hydrogen peroxide in presence of aniline with production of the same substance as is obtained by the action of tyrosinase on phenol and on catechol in presence of aniline. The peroxidase used was prepared by slicing horseradish into chloroform water, avoiding long exposure to air, and rejecting the outer portions. Only preparations were used which remained practically unchanged by aniline water in presence of hydrogen peroxide. 2 g. catechol and 4 cc. aniline were made up to nearly a litre, about 300 cc. peroxidase and 70 cc. 1 % hydrogen peroxide were added. A red precipitate appeared very rapidly. The product was treated in the same way as that obtained from phenol. Yield, 1·15 g. Properties: identical with those of the product obtained from phenol; M.P. 193°. Mixed M.P. with the product obtained from phenol, 193°; with that from catechol by tyrosinase, 192·5°.

Analysis. Found: N, 9·63 %.
Calculated for $C_{18}H_{14}O_2N_2$: N, 9·66 %.

*Synthesis of dianilino-*o*-benzoquinone.*

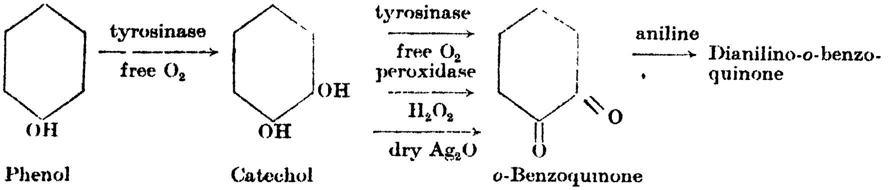
o-Benzoquinone in ethereal solution was synthesised by the method of Willstätter and Müller [1908] using 2·5 g. catechol. The ethereal solution was poured into a solution of 6 cc. aniline in 1700 cc. water, and shaken. A red precipitate separated out, which was filtered off. It was treated in the same way as the product obtained from phenol. Properties: identical with those of the products obtained from phenol and catechol; M.P. 193·5°. Mixed M.P. unchanged.

Analysis. Found: C, 74·47 %; H, 5·05 %; N, 9·67 %.
Calculated for $C_{18}H_{14}O_2N_2$: C, 74·48 %; H, 4·83 %; N, 9·66 %.

This proves the identity of the products obtained by enzyme action with dianilino-*o*-benzoquinone, and indicates therefore that *o*-benzoquinone is produced from phenol and from catechol by the action of tyrosinase, and from catechol by the action of peroxidase and hydrogen peroxide.

Dianilino-*o*-benzoquinone has been synthesised by Kehrmann and Cordone [1913] by oxidising catechol with silver oxide in glacial acetic acid solution containing aniline, the product being poured into water. The properties described are identical with the above.

The reactions taking place in the experiments described above may therefore be represented as follows:



Action of tyrosinase on p-*cresol in presence of aniline.*

6 g. *p*-cresol, 15 cc. aniline, and 100 cc. tyrosinase solution were made up to about a litre in buffered solution and aerated. The liquid became reddish, then a brownish red precipitate appeared. The major part of the substance remained too finely divided to be filtered off, so it was coagulated, or else extracted, as described for the filtrate in the case of phenol. The product was washed, dried, and extracted and crystallised by means of acetone. Some tarry substance was produced besides the anilinoquinone. The extraction was therefore fractionated; the tar, being exceedingly soluble in acetone, was removed in the earlier portions of the extract. Yield, about 1 g. of crystals. Properties: brownish red needles; M.P. 202°; soluble in organic solvents, but much less soluble in light petroleum than the product obtained from the lower homologue; soluble in concentrated sulphuric acid, giving a brownish crimson colour, becoming brown then yellow on dilution; insoluble in alkali.

Analysis. Found: C, 79·15 %; H, 5·55 %; N, 10·92 %.

Calculated for dianilinohomoquinoneanil, $C_{25}H_{21}ON_3$:

C, 79·15 %; H, 5·54 %; N, 11·08 %.

Action of tyrosinase on m-*cresol in presence of aniline.*

6 cc. *m*-cresol, 15 cc. aniline, and 100 cc. tyrosinase solution were made up to about a litre in buffered solution, and aerated. The product was treated in the same way as that obtained from *p*-cresol. Yield, less than 1 g. owing to the tarry nature of part of the extract. Properties: identical with those of the product obtained from *p*-cresol; M.P. 201·5°. Mixed M.P. unchanged.

Analysis. Found: C, 78·75 %; H, 5·57 %; N, 11·01 %.

Calculated for $C_{25}H_{21}ON_3$: C, 79·15 %; H, 5·54 %; N, 11·08 %.

Action of tyrosinase on homocatechol in presence of aniline.

The homocatechol used was prepared from creosol by the method of de Vries [1909], the creosol being prepared from vanillin. 1 g. homocatechol, 3 cc. aniline, and 40 cc. tyrosinase solution were made up to 150 cc. in buffered solution and aerated. The action was rapid and the yield remarkably good—about 0·9 g.—very little tar being obtained. Properties identical with those of the product obtained from *p*-cresol; M.P. 202°. Mixed M.P. with synthetic product (*vide infra*), 202°.

Analysis. Found: N, 10·80 %.

Calculated for $C_{25}H_{21}ON_3$: N, 11·08 %.

Action of peroxidase and hydrogen peroxide on homocatechol in presence of aniline.

0·5 g. homocatechol and 1·5 cc. aniline were dissolved in about 100 cc. buffer solution, and 90 cc. peroxidase and 18 cc. 2 % H_2O_2 added. The liquid became red and clouded, but no precipitate appeared. It was extracted with ether, the extract was dried over anhydrous sodium sulphate, filtered, and the filtrate evaporated, crystals being obtained which were recrystallised from acetone. Properties: identical with those of the product obtained from *p*-cresol; M.P. 201°. Mixed M.P. with the product obtained from *m*-cresol, 201°.

Analysis. Found: N, 11·04 %.
Calculated for $C_{25}H_{21}ON_3$; N, 11·08 %.

Synthesis of dianilinohomoquinoneanil.

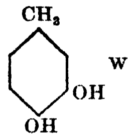
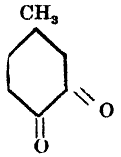
The following method was found to obviate the production of tar. Homoquinone in ethereal solution was synthesised by the method of Willstätter and Müller [1911], using 6 g. homocatechol. The solution was dropped slowly into a solution of 20 cc. aniline in water, mechanically stirred. The liquid became purplish, then almost black. On standing overnight, it became cloudy and brick red, and deposited a tarry solid. The lumps of solid matter which had separated were washed with a little ether and crystallised from acetone. A further yield was obtained by extracting the liquid with ether. This extract was dried over anhydrous sodium sulphate, filtered, and the filtrate evaporated on the water-bath, then left to stand in the vacuum desiccator, when crystals were deposited which were recrystallised from acetone. Total yield, about 2·5 g. Properties: identical with those of the products obtained from *p*-cresol, *m*-cresol, and homocatechol; M.P. 202·5°. Mixed M.P. unchanged.

Analysis. Found: C, 79·01 %; H, 5·67 %; N, 11·35 %.
Calculated for $C_{25}H_{21}ON_3$: C, 79·15 %; H, 5·54 %; N, 11·08 %.

The synthesis of dianilinohomoquinoneanil was also carried out by oxidising homocatechol with silver oxide in glacial acetic acid in presence of aniline, the product being poured into water. A method was also used analogous to that of Jackson and Koch [1898] for the preparation of dianilinoquinoneanil; homoquinone with aniline in chloroform was boiled on the water-bath for 10 minutes. The substances obtained by both these methods were identical with that obtained by the action of an ethereal solution of homoquinone on aniline water.

The identity of the products obtained by enzyme action with dianilinohomoquinoneanil is thus proved, and this indicates that homoquinone is produced from *p*-cresol, *m*-cresol, and homocatechol by the action of tyrosinase, and from homocatechol by the action of peroxidase and hydrogen peroxide.

The reactions taking place in the experiments just described may therefore be represented in a precisely similar manner to those with phenol (p. 1377).

m- and *p*-Cresol yield the same homocatechol  which then passes into homoquinone  and dianilinohomoquinoneanil.

The action of tyrosinase on other phenols.

The action of tyrosinase was tried on a number of other phenolic substances alone and in the presence of aniline. The following observations were made at p_H 6·5 or 7·0 after a period of 1 day, the phenols used being at concentrations of about 0·2 % or 0·1 % or, if only very slightly soluble, in saturated solution.

Substrate used	Substrate in buffer alone	Substrate in buffer with tyrosinase	Substrate in buffer with tyrosinase and aniline water	Substrate in buffer with aniline water
Quinol* (p_H 7·0)	Pink	Dark brown	Yellow	Yellow
,, (p_H 6·0)	Faintly pink	,,	,,	,,
Pyrogallol	Pale yellow	Darkened Brownish ppt. giving purpurogallin reaction with ammonia	Darkened Brownish ppt. No anilinoquinone	Pale yellow
m-Methoxyhydroxybenzene	Nil	Reddish brown	Bright red ppt. of anilinoquinone	Very faintly coloured
p-Methoxyhydroxybenzene	Nil	Reddish brown	Bright red ppt. of anilinoquinone	Very faintly coloured
p-Aminophenol	Brown ppt.	Brown ppt. Darkened	Brown ppt. Darkened	Brown ppt.
Tyrosine	Nil	Dark brown	Brown. No anilinoquinone	Nil

* The oxidation of quinol appears to be inhibited unless the solution is dilute and the enzyme very active. A similar phenomenon may possibly occur with guaiacol. The matter has not yet been fully investigated. The yellow colour with quinol and aniline is due to an addition compound.

No reactions were observed with the following: resorcinol, phloroglucinol, guaiacol, *o*-cresol, orcinol, creosol, eugenol, *iso*-eugenol, *o*-, *m*-, and *p*-nitrophenol, and thymol. Vanillin and salicylaldehyde reacted as aldehydes with aniline, the other tests being negative.

Thus the only further substances found to give rise to anilino compounds were *m*- and *p*-methoxyhydroxybenzene, which were attacked apparently in an analogous manner to *m*- and *p*-cresol. In presence of aniline bright red substances were obtained, having the properties of an anilinoquinone: they were soluble in concentrated sulphuric acid, giving a brownish colour, reprecipitated on dilution; soluble in alkali, giving a purplish red solution, reprecipitated on acidification.

Action of tyrosinase on catechol in presence of benzenesulphinic acid.

The addition product of *o*-benzoquinone with benzenesulphinic acid was obtained by Hinsberg and Himmelschein [1896] by the oxidation of catechol in presence of benzenesulphinic acid.

An attempt was made to obtain this compound by the action of tyrosinase on phenol, and also on catechol, in presence of benzenesulphinic acid. The attempt was successful in the case of catechol, but not in that of phenol. *o*-Dihydroxydiphenylsulphone (M.P. 165°) was found to be very feebly soluble in benzene, in which phenol, catechol, and benzenesulphinic acid are all readily soluble. The product of action of tyrosinase on catechol in presence of benzenesulphinic acid was isolated by making use of these facts.

0·2 g. catechol and 0·3 g. benzenesulphinic acid were dissolved in water, and the p_H adjusted with phosphate buffer to 6·0–6·5. The solution was made up to 100 cc., 3 cc. tyrosinase solution were added, and the liquid was allowed to stand for a few days, with frequent shaking. It was then extracted with ether and the ether evaporated. The residue was boiled under reflux with benzene for a minute or two and the white residue filtered off. This was dissolved in boiling water, from which it separated as crystals on cooling. The crystals were dried in a desiccator over concentrated sulphuric acid *in vacuo*, water of crystallisation being thereby removed. Properties: white powder, M.P. 164°; mixed M.P. with *o*-dihydroxydiphenylsulphone, unchanged; aqueous solutions of both gave a blue-green colour with ferric chloride, changed to red by sodium carbonate.

Several attempts, including the use of increased aeration, were made to obtain the same substance by the action of tyrosinase on phenol in presence of benzenesulphinic acid; but in every case the residue on evaporation of the ether was readily entirely soluble in benzene in the cold. Thus there is no evidence that tyrosinase will oxidise phenol in presence of benzenesulphinic acid. It is possible that a higher oxygen potential is required for the oxidation by tyrosinase of phenol than of a catechol, and that the reducing action of benzenesulphinic acid prevents this potential from being reached. But this can be determined only by further experiment.

Deamination of glycine by homoquinone.

The method used was essentially that described by Happold and Raper [1925], a funnel being used to run in the solutions, and homoquinone taken in place of *o*-benzoquinone. 0·2 g. solid homoquinone dissolved in ether was run into 0·5 g. glycine in buffer at p_H 7·0. The reaction mixture was aerated gently for a day, any ammonia escaping in this process being absorbed in $N/20$ H_2SO_4. The whole of the ammonia produced was finally driven over into the absorption bottles by running into the reaction bottle 1·5 g. sodium carbonate in water, the stream of air being then continued for several hours.

The amount of acid neutralised by ammonia produced during the reaction corresponded to 0·06 g. homoquinone, *i.e.* 30 % of the homoquinone used, the reaction

$$CH_3.C_6H_3O_2 + NH_2.CH_2.COOH + H_2O = NH_3 + CO_2 + H.CHO + CH_3.C_6H_5O_2$$

being assumed. Another experiment gave 34 %.

The homoquinone does not, therefore, yield the equivalent of ammonia. This may be due partly to combination of the ammonia and formaldehyde to give hexamethylenetetramine, and partly to other reactions which lead to the production of highly coloured substances, since during aeration the liquid in the reaction bottle became deeply coloured, being blue by transmitted, red by reflected light. It was similar to a liquid obtained by the action of tyrosinase on *p*-cresol in presence of glycine (Chodat's reaction). Both on addition of alkali became reddish, and on addition of acid blue.

Measurements of oxygen absorption.

In most cases colour changes in the solution, and in some cases the formation of an anilinoquinone in presence of aniline, serve to determine whether a given phenol is oxidised by tyrosinase or not. In certain cases recourse was had to a microrespirometer of the Barcroft type, in which carbon dioxide was absorbed. The following pairs of mixtures were shaken side by side. Respirometer I. Left: the phenol used; phosphate buffer; tyrosinase. Right: buffer; tyrosinase. Respirometer II. Left: the phenol used; buffer. Right: buffer alone. Any autoxidation taking place could thus be detected. At the commencement of each experiment, the apparatus was equilibrated by shaking for 5 minutes with the taps open; the results obtained are therefore minimum values, since in some cases a small amount of oxygen would be absorbed during equilibration. The quantity of phenol used was 1 or 2 mg. Solid phenylurethane or thymol was added to each flask as antiseptic, the enzyme used being freed from chloroform *in vacuo*. The activity of the enzyme at the end of the experiment was tested by addition of *p*-cresol to the left flask of Respirometer I.

The amounts of oxygen absorbed by phenols attacked by tyrosinase were measured in this way, and calculated in terms of atoms of oxygen per molecule of phenol, some indication being thus obtained of the extent of oxidation taking place. The following substances were examined in the respirometer,

Substrate	p_H	Atoms of oxygen absorbed per molecule of substrate	
Phenol	7·6	3·4 +	
o-Cresol	7·6	Nil	
m-Cresol	7·0	2·9	
p-Cresol	7·6	2·6	
,, ...	7·8	3	Robinson and McCance
Catechol	7·8	2	
Resorcinol	7·0	Nil	
Quinol	6·0	3·0	
Pyrogallol	6·0	1·5	
m-Methoxyhydroxybenzene (slightly impure)	7·0	2 +	
p-Methoxyhydroxybenzene	7·0	3·0	

with the results stated. Two complementary results obtained by Robinson and McCance [1925] are included.

p-Aminophenol hydrochloride, brought to p_H 6·0 with NaOH, and buffered, showed autoxidation, but a definite increase in oxygen absorption in presence of tyrosinase. Thymol was also tested, its absorption being nil.

The figure 1·5 for pyrogallol was obtained after deduction of slight absorption due to autoxidation. The absorption of three atoms of oxygen per two molecules of pyrogallol corresponds to the formation of purpurogallin.

The experiments of Robinson and McCance [1925], in which slight oxygen absorption was obtained with resorcinol in presence of tyrosinase and a delayed but considerable absorption when glycine was also present, were repeated, but no confirmation of their results was obtained. In most cases the enzyme was inactivated by resorcinol after a few days, but in a case in which it remained active no absorption was observed.

Catechol, *m*- and *p*-cresol, *m*- and *p*-methoxyhydroxybenzene all show an absorption per molecule of one atom of oxygen more than that corresponding to formation of *o*-quinone. Phenol shows an even greater absorption. This appears to indicate that some further reaction involving oxidation occurs. In absence of some substance with which they combine, the *o*-quinones appear to undergo further change almost at once. They are found merely in traces (as judged by the aniline reaction) when the action of the enzyme on the phenol has been allowed to go to completion.

SUMMARY.

1. Anilino-*o*-quinones produced by the action of tyrosinase on several phenols in presence of aniline have been isolated. The substances obtained from phenol and catechol were identical. The substances obtained from *m*-cresol, *p*-cresol, and homocatechol were also identical. This indicates clearly that *o*-quinones are produced by the action of tyrosinase on these phenols.

2. Peroxidase and hydrogen peroxide, acting on catechol or homocatechol, also produce *o*-quinones.

3. Further evidence indicating the formation of *o*-quinones consists in the isolation of a sulphinic acid derivative of *o*-benzoquinone by the action of tyrosinase on catechol in presence of benzenesulphinic acid.

4. Homoquinone deaminates glycine in the same manner as does *o*-benzoquinone.

5. *m*- and *p*-Methoxyhydroxybenzene were the only phenols, in addition to those mentioned above, which were found to give rise to anilinoquinones.

6. The oxygen absorbed during the oxidation by tyrosinase of several phenols has been measured, indications being obtained with those phenols giving rise to anilinoquinones that, in absence of aniline, some further reaction involving oxidation occurs beyond formation of the *o*-quinone.

7. The "direct oxidase" theory of Bach and Chodat is criticised in the light of the above results, and a new classification of oxidases into aerobic and anaerobic oxidases is suggested.

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CLXXXI. DISSOCIATION CONSTANTS OF CYSTINE, CYSTEINE, THIOGLYCOLLIC ACID AND α-THIOLACTIC ACID.

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Mathews and Walker [1909] and, in greater detail, Dixon and Tunnicliffe [1923] have demonstrated the great significance of the hydrogen ion activity in relation to the rates of oxidation and of autoxidation of a number of thio-acids. To assist the analysis of these results and of certain observations of our own it became desirable to know the dissociation constants of cysteine, thioglycollic acid and thiolactic acid. It was thought worth while at the same time to determine the corresponding constants of cystine in order that a gap in the electrochemical data on the natural amino-acids might be filled.

With respect to cysteine, thioglycollic acid and thiolactic acid no difficulty was encountered in obtaining reproducible hydrogen electrode titration curves from which the several constants could be deduced. The method was, however, not available in the case of cystine as this substance suffers reduction at the hydrogen electrode surface with consequent depolarisation of the electrode. The quinhydrone electrode suggested itself as an alternative since its potential is positive to that which, on chemical and other grounds, may reasonably be attributed to solutions of cystine. This electrode, indeed, proved satisfactory within its limitations but was rejected because it does not permit the extension of a titration to the low concentrations of hydrogen ions at which stages in the dissociation of cystine occur.

An oxidation-reduction electrode was sought, therefore, which would be available over the whole significant p_H range and whose equilibrium would not be disturbed by the presence of cystine. The methylene blue-methylene white electrode fulfilled these requirements.

Experimental.

The titration vessel used for the hydrogen electrode titrations was similar to that employed by Clark and Cohen [1923] for oxidation-reduction titrations. This carried a pair of palladium-coated, gold-plated platinum electrodes and a supply tube for hydrogen gas. A saturated calomel electrode and a saturated potassium chloride bridge completed the cell, which was immersed in a kerosene thermostat at 30°. The E.M.F. was determined potentiometrically against a standard Weston cell. At no significant points on the titration curves did the two electrodes differ by more than 0·1–0·3 mv. In the type of cell employed the hydrogen stream is delivered through the solution but not directly over the electrode surfaces. Consequently the initial attainment of equilibrium at the electrode occupies some time after the introduction of the solution to be titrated: 20–30 minutes was found adequate. Subsequent adjustments to additions of titrating agent are rapid, and true equilibrium values can be determined in 2–3 minutes after each addition.

For our own purposes we required to know the p_H of approximately 0·05 molar solutions of the sulphydryl compounds neutralised to varying degrees with 0·5 molar CO_2-free sodium hydroxide. For this reason the electrometric titrations were performed with solutions of similar strength. It was appreciated that the constants derived from observations on solutions of such ionic strength are subject to a significant correction for activity: No such correction has been applied because, in the case of the two ampholytes, its calculation is uncertain, and because most of the data in the literature on the constants of the natural amino-acids are uncorrected values (K') referable to solutions of about 0·1 molar strength. Some indication of the magnitude of the activity correction was obtained by repeating the titrations on 0·01–0·02 molar solutions. A further advantage in working at fairly high concentration is the more extensive data which can be obtained on the constants functioning at the extremes of the p_H scale.

Cystine was prepared by the acid hydrolysis of hair and recrystallised several times. From this, cysteine hydrochloride was obtained by reduction with zinc and hydrochloric acid, precautions being taken against re-oxidation during crystallisation. Thioglycollic acid (B.P. 109–110°/16 mm.) was prepared from monochloroacetic acid and sodium disulphide followed by reduction of the dithiodiglycollic acid with zinc and hydrochloric acid. α-Thiolactic acid (B.P. 107–110°/17 mm.) was prepared by the method of Lovén [1884] from α-chloropropionic acid. These acids were redistilled at low pressure in nitrogen.

The titration curves are assembled in Fig. 1, in which the "corrected equivalents of base" [Simms, 1926] are plotted against the observed p_H values.

Table I, which is later explained, records some of the data from which the curve for cysteine has been constructed and serves to exhibit the order of consistency attained throughout the work.

It is apparent, in respect of thioglycollic acid and thiolactic acid, that the two constants differ so greatly in magnitude that the curve may be treated as that of a mixture of two monobasic acids, whose constants are derived from the relation

$$p_{K'} = p_H - \log \frac{a}{1-a}.$$

Table I. *Titration of* 0·1 *M cysteine hydrochloride with M sodium hydroxide.* 30°.

$p_{K_1'} = 1{\cdot}86$ $p_{K_2'} = 8{\cdot}14$ $p_{K_3'} = 10{\cdot}34$

p_H	a_1	a_2	a_3	Corrected equivalents of base Calculated	 Found
1·626	0·368	—	—	0·368	0·343
1·675	0·395	—	—	0·395	0·379
1·841	0·489	—	—	0·489	0·485
2·030	0·597	—	—	0·597	0·596
2·151	0·661	—	—	0·661	0·675
2·340	0·769	—	—	0·769	0·755
2·860	0·909	—	—	0·909	0·907
7·000	One equivalent complete	0·0676	0·0000	1·068	1·081
7·607		0·2266	0·0004	1·227	1·234
8·046		0·4450	0·0023	1·450	1·447
8·422		0·6516	0·0079	1·667	1·660
8·887		0·8236	0·0290	1·882	1·872
9·200		0·8624	0·0625	1·988	1·979
9·545		0·8334	0·1336	2·101	2·106
9·965		0·6962	0·2935	2·283	2·298
10·160		0·5987	0·3955	2·390	2·404
10·520		0·3971	0·6011	2·599	2·610
10·756		0·2771	0·7219	2·721	2·712
11·035		0·1679	0·8320	2·832	2·798
11·595		0·0526	0·9475	2·948	2·934

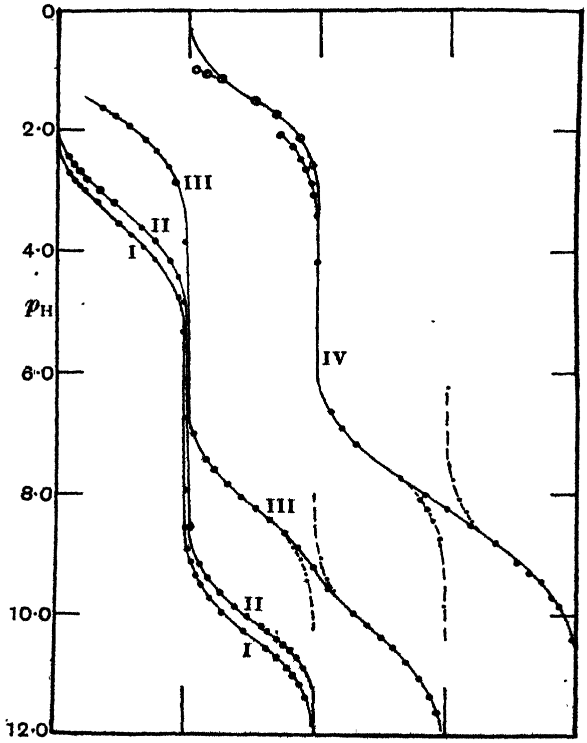
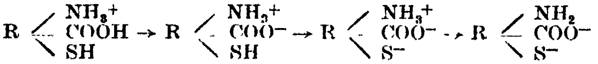


Fig. 1.

Curve I = α-Thiolactic acid. Curve II = Thioglycollic acid. Curve III = Cysteine.
Curve IV = Cystine { ●—●—● M/50 M. blue electrode. ○—○—○ M/10 Quinhydrone electrode.

The case of cysteine is more complex. To avoid the necessity for discriminating between acidic and basic constants in the analysis it is of assistance to regard the kation of the hydrochloride as an acid dissociating three hydrogen ions. The constants K_1', K_2', K_3' refer to the successive steps without any present implication as to the specific group which determines the value of each constant.



$$R\begin{cases}NH_3^+\\COOH\\SH\end{cases}\rightarrow R\begin{cases}NH_3^+\\COO^-\\SH\end{cases}\rightarrow R\begin{cases}NH_3^+\\COO^-\\S^-\end{cases}\rightarrow R\begin{cases}NH_2\\COO^-\\S^-\end{cases}$$

It is clear from the curve that the value of K_1' is so much greater than those of K_2' and K_3' that it may be derived separately. The influences of K_2' and K_3', however, significantly overlap. This region of the curve was therefore treated as that of a dibasic acid. Graphic approximations to the values of $p_{K_2'}$ and $p_{K_3'}$ were made and the values of α_2 (primary ion) and α_3 (secondary ion) were calculated from the equations

$$\alpha_2 = \frac{1}{1 + \frac{[H^+]}{K_3'} + \frac{K_3'}{[H^+]}}.$$

$$\alpha_3 = \frac{1}{1 + \frac{[H^+]}{K_3'} + \frac{[H^+]^2}{K_2' K_3'}},$$

which are derived from the application of the mass law [Michaelis, 1921]. Thence were calculated the equivalents of base $(1 + \alpha_2 + 2\alpha_3)$ required to bring to the observed p_H values a mixture of three acids having the given constants. These values are compared with the corrected equivalents of base derived from the titration readings. Minor modifications of the graphic values of $p_{K'}$ were then made to improve the alignment of the above data[1].

Methylene blue-methylene white electrode.

Clark, Cohen and Gibbs [1925] have found that the electrode potential of a given ratio of the oxidised and reduced forms of this system varied not only with different samples of the dye but also with the total concentration present. Fortunately we were able to overcome these difficulties through the gift of a specimen of Sample *F* of the methylene blue for which Clark, Cohen and Gibbs have recorded electrode data. The dye was employed at a similar concentration to that with which they worked (0·0001 *M* total dye). Into a burette, protected from oxygen, was introduced a solution, approximately equimolar, of methylene blue and methylene white. The potential of this mixture at a gold-plated platinum electrode at p_H 3·97 was determined by adding 5 cc. to 35 cc. of 0·057 *M* potassium acid phthalate.

[1] As Simms [1926] has pointed out, these constants are titration constants (G') and are related to the true dissociation constants (K') by the equations

$$K_1' = G_1' + G_2',$$

$$\frac{1}{K_2'} = \frac{1}{G_1'} + \frac{1}{G_2'}.$$

The correction is negligible in the present case.

The potential of the methylene blue-methylene white electrode is related to $[H^+]$ by the following equation [Clark, Cohen and Gibbs, 1925] (which holds acid to p_H 12):

$$E_h = C + 0{\cdot}03006 \log [K_1K_2 [H^+] + K_2 [H^+]^2 + [H^+]^3]$$

where E_h is the potential referred to the normal hydrogen electrode,

$$K_1 = 1{\cdot}4 \times 10^{-6} \text{ and } K_2 = 3{\cdot}0 \times 10^{-5},$$

being dissociation constants of methylene white.

C is a constant given by

$$C = 0{\cdot}533 - 0{\cdot}03006 \log \frac{[\text{methylene white}]}{[\text{methylene blue}]}.$$

For any experimental ratio of the oxidised and reduced forms C can be calculated by determining the potential (E_h) at a known p_H. With this determined the equation may be used to relate observed potentials of the given mixture to the p_H of the solution to which it has been added. The equation is cumbersome, however, and it is convenient that K_1 and K_2 are of such an order that within the range p_H 1–2·5 the relation reduces to $E_h = C - 0{\cdot}09018\ p_H$; and between p_H 7·8–12·00 to

$$E_h = C - 0{\cdot}313 - 0{\cdot}03006\ p_H.$$

In view of the low solubility of cystine the titration was carried out on a 0·02 molar solution containing sufficient standard hydrochloric acid to effect solution. To 35 cc. of this solution were added 5 cc. of the dye mixture and the titration was conducted in purified nitrogen with oxygen-free sodium hydroxide. The excellent agreement between the potentials of two electrodes, their reproducibility, the absence of potential drifts, and the satisfactory analysis to which the data submitted gave assurance that the electrode gave a true measure of the p_H. No precipitation of the cystine was observed at any stage of the titration.

In the case of cystine there are four constants to be determined. By analogy with cysteine and other amino-acids it will be expected that one pair will be active in the acid range and one on the alkaline side of neutrality. The curve shows this to be so and permits each pair of constants to be derived by treating their section of the curve as that of a dibasic acid, as was done in the case of cysteine. As a result it appears that K_1' is too great to be measured. K_2', K_3' and K_4' have been deduced. K_2' was determined also by titration of a 0·1 molar solution of cystine hydrochloride at the quinhydrone electrode. Precipitation of cystine did not occur, even at this concentration, until the titration of the first two equivalents was almost complete.

The third and fourth titration constants lie so close together that they differ slightly from the true constants. Applying the correction of Simms [1926] (footnote, p. 1387) we get for the uncorrected apparent dissociation constants the values in Table II.

Table III is an abbreviation of the data for K_3' and K_4' constructed in the same way as Table I. It serves to show the order of consistency of the methylene blue electrode readings.

Table II. *Apparent dissociation constants* (K') *at* 30°: *not corrected for activity.*

	Approx. molar strength	$p_{K_1'}$	$p_{K_2'}$	$p_{K_3'}$	$p_{K_4'}$	K_1'	K_2'	K_3'	K_4'
Thioglycollic acid	0·1	3·4	10·0	—	—	4×10^{-4}	1×10^{-10}	—	—
	0·01	3·5	10·2	—	—	3×10^{-4}	6×10^{-11}	—	—
Thiolactic acid	0·025	3·6	10·3	—	—	$2{\cdot}5 \times 10^{-4}$	5×10^{-11}	—	—
	0·01	3·7	10·3	—	—	$2{\cdot}0 \times 10^{-4}$	5×10^{-11}	—	—
Cysteine	0·1	1·86	8·14	10·34	—	$1{\cdot}4 \times 10^{-2}$	$7{\cdot}3 \times 10^{-9}$	$4{\cdot}6 \times 10^{-11}$	—
	0·02	1·96	8·18	10·28	—	$1{\cdot}1 \times 10^{-2}$	$6{\cdot}6 \times 10^{-9}$	$5{\cdot}3 \times 10^{-11}$	—
Cystine	0·1	<1·0	1·5	—	—	$>1 \times 10^{-1}$	$3{\cdot}2 \times 10^{-2}$	—	—
	0·02	<1·0	1·7	7·48	9·02	$>1 \times 10^{-1}$	$2{\cdot}0 \times 10^{-2}$	$3{\cdot}3 \times 10^{-8}$	$9{\cdot}6 \times 10^{-10}$

Table III. *Titration of* 0·02 *M cystine hydrochloride with* 0·1 *M sodium hydroxide.* 30°.

$p_{K_1'} = < 1{\cdot}00$ $p_{K_2'} = 1{\cdot}72$ $p_{K_3'} = 7{\cdot}48$ $[pG_3' = 7{\cdot}50]$ $p_{K_4'} = 9{\cdot}02$ $[pG_4' = 9{\cdot}00]$

					Corrected equivalents of base	
p_H	a_1	a_2	a_3	a_4	Calculated	Found
2·26	Assume 1 equivalent conpleted	0·776	—	—	1·776	1·792
2·47		0·849	—	—	1·849	1·853
2·63		0·889	—	—	1·889	1·887
2·87		0·935	—	—	1·935	1·935
3·07		0·957	—	—	1·957	1·957
6·62	2 equivalents completed		0·1164	0·0005	2·117	2·117
6·90			0·2005	0·0016	2·204	2·206
7·15			0·2611	0·0035	2·268	2·296
7·44			0·4591	0·0127	2·484	2·474
7·72			0·6039	0 0317	2·667	2·652
8·02			0·7109	0 0745	2·870	2·830
8·25			0·7375	0 1342	3 006	3·010
8·52			0·7008	0·2321	3·165	3·188
8·81			0·5899	0·3810	3·352	3·366
9·12			0·4267	0·5628	3·552	3·542
9·30			0·3316	0·6600	3·652	3·630
9·45			0·2609	0·7225	3·706	3·718
9·71			0·1793	0·8360	3·851	3·804
9·85			0·1412	0·8757	3·893	3·854
10·41			0·0375	0·9627	3·963	3·946

Remarks.

The treatment which has been accorded the dissociation of cysteine and cystine has not distinguished acidic and basic constants. It is still a common custom to refer the first dissociation constant of amino-acids to the basic group and the second to the acid group, *i.e.*, an amino-acid is regarded as both a weak acid and a weak base. On this basis the K_1' here reported for cysteine, and the K_1' and K_2' for cystine would be the hydrolysis constants of the NH_2 groups related to K_b by $K_b = \frac{Kw}{K'}$.

Adams [1916] and Bjerrum [1923] on the other hand have adopted the more plausible view that the constant active at an acid reaction is the acidic constant and that functioning at low [H^+] is referable to the NH_2 group. In the case of cystine, therefore, K_1' and K_2' relate to the symmetrical COOH groups and K_3' and K_4' become the hydrolysis constants of the NH_2 groups. The enhanced values of the acid constants and the diminution in strength of the basic groups in comparison with other amino-acids is to be remarked. In the case of cysteine we may give K_1' to the carboxyl group but there is little to distinguish the influence of the sulphydryl- and amino-groups in the two stages of dissociation in the alkaline range. Comparison with the simple thio-acids which have been titrated would suggest that K_3' of cysteine is determined by the sulphydryl group. On the other hand the – SH group in cysteine is further removed from the COOH than in thioglycollic and in thiolactic acids, and should therefore be a stronger group. Moreover K_3' approximates much more closely to the values of the NH_2 constant of related amino-acids (*e.g.* alanine, serine) than does K_2'. It is probable that a plausible argument could be made for either allocation of K_2' and K_3' in cysteine. A decision seems premature and in any case can only be qualitatively true in view of the fact that in cases where two constants are of similar magnitude both groups involved are concerned in determining the value of each constant.

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CLXXXII. STUDIES IN THE METABOLISM OF TISSUES GROWING *IN VITRO*.

II. EFFECT OF GLUCOSE UPON THE AMMONIA AND UREA PRODUCTION OF KIDNEY TISSUE.

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(*Received October 31st, 1927.*)

In the first paper of this series [Holmes and Watchorn, 1927] we described the formation of ammonia and urea by the tissue of embryonic rat kidneys when this was growing *in vitro*. The formation of these substances occurred only when actual growth took place, and we were inclined to believe that it indicated the combustion of nitrogenous substances to supply the energy necessary for growth. If this were the case, the addition of glucose, *i.e.* the supplying of another and probably more available source of energy, might be expected to lessen the production of ammonia and urea during the growth of the tissue.

These experiments were undertaken to discover whether such an effect is produced by adding glucose to the culture medium.

Technique.

This has been essentially the same as that previously described [Holmes and Watchorn, 1927]. Some modifications of Stanford's ammonia method [1923] have been introduced. We have used much hotter water for surrounding the distilling flask—namely about 60°. If the apparatus has just been well evacuated, this enables brisk distillation to proceed for 12 minutes, or often longer, without the apparatus being attached to the filter pump. It should, however, be connected to the pump for two or three minutes before finally discontinuing distillation, otherwise a slightly low result is apt to be obtained. When letting down the vacuum, air should be admitted through a sulphuric acid wash-bottle. At no period during distillation do we pass air through the apparatus. If cooler water is used, as described by Stanford, attachment to the filter pump is necessary after about 3 minutes, and under these conditions we have found that usually only 90 % of the ammonia distils over. We have assured ourselves that, at any rate for our solutions, the hotter water is no disadvantage.

Glucose determinations have been made by Hagedorn and Jensen's [1923] method. Hydrogen ion concentration has been measured by aid of the B.D.H. capillator, using bromothymol blue as indicator, which completely covers our range.

EXPERIMENTAL.

Pannett and Compton's [1924] Ringer solution was used for diluting the embryo extract. Varying amounts of glucose have been added at different times. As we previously showed that no ammonia or urea was formed by resting tissue (*i.e.* tissue which was living, but was floating freely in the medium and thus unable to grow) after 48 hours' incubation at 37° we have frequently discarded the elaborate system of controls formerly used, and have simply taken the resting tissue preparations as controls for the growing tissues. Preliminary experiments with resting tissues have shown that when kidneys from the same litter are used, the addition of glucose to an otherwise similar medium does not affect the non-occurrence of ammonia or urea formation by non-growing, non-autolysing tissue (Table I).

Table I.

	Mg NH_3-N		Mg urea- + NH_3-N	
Exp.	Without glucose	*With glucose	Without glucose	*With glucose
20	0·036	0·034	0·060	0·061
22	0·032	0·032	0·062	0·061
29	0·048	(i) 0·046 (ii) 0·042	0·084	(i) 0·087 (ii) 0·083
30	—	—	0·056	(i) 0·053 (ii) 0·055
32	0·044	0·046	0·114	0·119
34	0·027	0·026	0·082	0·075

* The original concentration of glucose in these cases is from 0·07 to 0·09 %.

Having established this fact, it was then possible to use resting tissue preparations containing glucose as controls for growing tissue.

The addition of glucose gave rise to a marked alteration in the metabolism of the growing tissue, in that it caused a very definite inhibition of ammonia and urea formation. Unfortunately, as the number of embryos in a litter is sometimes small, it has not always been possible to include preparations of growing tissues in plain and in glucose media in the same series. This is not a very serious drawback, as we have previously obtained a large number of experimental results showing that ammonia and urea are formed when the tissue grows in the absence of glucose. In the presence of glucose the formation of these substances is, as is shown by comparison with the resting controls, frequently completely inhibited. Under these circumstances, when the results obtained are exactly similar in growing preparations and in controls, it is necessary to be certain that growth has actually taken place. Even if tissue without glucose is included in the same series, and is known to have grown well, there is obviously no certainty that the glucose-fed tissue has also grown, as the presence of the sugar may either inhibit or accelerate growth. Except in the early experiments, we have therefore included in each series one or more small control dishes containing small amounts of tissue growing in the same medium, which could be used for staining and microscopical examination. These revealed the fact that very small amounts of glucose in the medium did not inhibit, but if anything aided growth. In some cases better differentiation seemed to take place in the presence of glucose than in its absence. However, on one or two occasions when a concentration of over 0·2 % was present, inhibition of growth and even death of the tissue apparently took place[1]. This concentration is, of course, remarkably low compared with figures given for possible glucose concentrations by other observers [Lewis, 1922; Willmer, 1927], who find 1 % and even 2 % solutions suitable for growth. This difference may be due to the fact that we have used mammalian tissue, whereas other observers have dealt with chick tissue. The amount of glucose in the egg is fairly large at some stages of its development, and it is possible that the tissues of the embryo are exposed to relatively high concentrations. Indeed, the blood-sugar in embryo chicken blood is about 0·2–0·3 % at some stages of development [Hanan, 1925]. Willmer [1927] states that 0·2 % would be the best strength for the *in vitro* growth of chick intestine if it could be constantly maintained. In the case of embryo rat kidney we find that concentrations approaching mammalian blood-sugar level (*i.e.* 0·07 to 0·11 %) are more suitable.

Figures for tissues growing in a glucose medium are given in Table II. The concentration of glucose originally present was from 0·07 to 0·11 %.

Table II.

	Control		Growing tissue	
Exp.	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N
20	0·036	0·060	0·039	0·064
22	0·032	0·062	0·033	0·052
			0·024	0·051
29	0·048	0·084	0·042	0·069
			0·039	0·069
30	0·028	0·066	0·028	0·067
33	0·046	0·120	0·046	0·108
34	0·026	0·075	0·027	0·055
			0·029	0·072
35	0·028	0·055	0·026	0·057
36	—	0·079	—	0·080

It will be seen that, in some experiments, the growing tissue has the same figure for ammonia and urea as its control, but that in others (Exps. 22, 29, 33 and 34) the total ammonia- and urea-nitrogen is actually less in the growing preparation than in the resting tissue. It is perhaps significant that these were all flasks in which good growth had taken place. When tissues grown in plain and in glucose media are included in the same series (that is to say, when all kidneys used are taken from the same litter, and the media made from the same embryo extract) then the results are even more striking (Table III).

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[1] The glucose solutions used in these cases had been autoclaved, and their toxicity may be due to this.

Table III.

	Resting control		Tissue growing in absence of glucose		Tissue growing in presence of glucose		Increase in NH_3- + Urea-N	
Exp.	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N	NH_3-N	NH_3- + Urea-N	Without glucose	With glucose
20	0·036	0·060	0·050	0·079	0·039	0·064	0·019	Trace
			0·075	0·075			0·015	
28	0·045	0·076	0·060	0·105	—	0·092	0·029	0·016
30	0·028	0·066	0·050	0·115	0·028	0·067	0·049	Nil
35	0·028	0·055	0·027	0·084	0·026	0·057	0·029	Nil
				0·085			0·030	

The concentrations of glucose originally present vary from 0·07 to 0·11 %.

In Exp. 30, for instance, which is shown in this table, the growth in the glucose medium was particularly good, yet the content of ammonia- and urea-nitrogen showed no rise, whereas in the plain medium the growth of the tissue caused a 74 % increase in the amount of ammonia and urea present. Exp. 35 in Table III gives another very definite illustration of the effect of the addition of glucose.

We had hoped when this work was first begun that we might find a striking quantitative difference in the carbohydrate metabolism of the floating (resting) and the growing tissues. Up to the present we have only attempted to estimate the disappearance of glucose, and the results have been somewhat disappointing, as there is often no such difference found. As might have been expected from the work of Warburg and others, the resting kidney tissue itself is active in breaking down glucose; thus, anything which delays the commencement of growth or diminishes its extent must tend to obscure any quantitative differences between the resting and the growing preparations. Again, estimations showing the disappearance of glucose from the medium give no indication of the manner in which the sugar may be utilised. It is possible, for instance, that the breakdown of the glucose may be far more complete, and the energy obtained therefore far greater in amount, in some preparations than in others. It may even happen that further breakdown of glucose occurs after the flasks are removed from the incubator, and we have taken no special precautions to prevent this.

Although in many cases the resting tissue has caused as much glucose to disappear as the growing tissue, it has been possible in experiments where very good growth has occurred to demonstrate an extra glucose breakdown on the part of the growing tissue. Thus, in Exp. 30, the original amount of glucose present in each flask was 1·5 mg., the amount remaining in the control preparation 0·9 mg., and the amount remaining in the growing preparation only 0·7 and 0·4 mg. The glucose estimations are carried out on small portions of the filtrates in which the ammonia and urea values are determined. A control flask is kept at – 2° for the duration of the experiment and is used for estimation of the amount of glucose originally present; we have usually found that about one-third of this amount is broken down by the resting tissue.

p_H determinations have also been carried out on the filtrates. These have been found to give a rough check on the figures we obtain for glucose breakdown, and are more generally useful in determining the optimum hydrogen ion concentration for tissues growing under our particular conditions.

Discussion.

We had previously shown that kidney tissue which was growing in a plain embryo extract always formed considerable amounts of urea and ammonia, though resting (floating) tissue under identical conditions did not. We have now found that, in the presence of glucose, very good growth may occur without any production of urea and ammonia by the growing tissue.

It seems probable, therefore, that, in the absence of glucose, nitrogenous substances are broken down to provide the energy necessary for the growth of the tissue, thus giving rise to the ammonia and urea which appear. When glucose is present the necessary energy may be obtained instead from the breakdown of glucose.

In a great many cases we have obtained very good growth in glucose media without any increase (sometimes even a slight decrease) in the ammonia- and urea-nitrogen. Sometimes a slight but definitely lessened production of these substances is found, while on one occasion the increase in ammonia- and urea-nitrogen seemed to be unaffected by the presence of the glucose, although it is probable that in this case the extent of the growth was greater in the glucose medium than in the plain.

We have no explanation for these variations, which may be due to differences in the age of the embryos, alterations in the glucose during sterilising, or varying proportions in the media of glucose and the nitrogenous substances which are broken down by the tissue. There is no doubt, however, that we have obtained very definite evidence of the so-called "protein-sparing" action of glucose.

Raistrick [1921], dealing with the effect of glycerol upon the breakdown of tryptophan by bacteria, claims that glycerol cannot be said to "spare" the amino-acid, since, although less ammonia is formed when the glycerol is present, a larger amount of the nitrogen from the tryptophan is built up into the bacterial tissues. Raistrick considers that the effect of the addition of glycerol is to produce a more suitable carbon/nitrogen ratio for bacterial growth. Even in this case, however, there was (this was pointed out to us by Miss M. Stephenson) more tryptophan left untouched at the end of the experiment when the bacteria were grown in the glycerol medium. than when they were grown in a medium containing tryptophan alone, so that some actual sparing of the amino-acid did occur. We are inclined to believe that in our case the glucose has much this effect, since, although the oxidative breakdown of nitrogenous substances (or that part of it which is manifested by the appearance of ammonia and urea) is largely prevented, the growth is probably greater in extent, and therefore more nitrogen must be removed from the medium for purposes of synthesis.

Lewis [1922], Willmer [1927] and others have employed glucose in culture media, and consider it useful in maintaining growth. Lewis, in fact, finds that it much prolongs the life and activity of cells which are allowed to grow for some time in the same drop of culture medium. It will be seen from consideration of the facts set forth in this paper that this prolongation of life and the power of growth may be due to the addition of extra carbohydrate fuel, or to the prevention of the accumulation of ammonia and possibly other products of protein breakdown. It is highly probable that unduly high concentrations of ammonia are very toxic for tissue cells. This is in fact indicated by the work of Krontovski and Radzimovska [1922] who showed that exposure of tissues for half an hour to Michaelis' ammonia standards inhibited the subsequent growth of the cells far more than the alkalinity of the solutions warranted.

In a previous paper [Holmes and Watchorn, 1927] we discussed the apparent contradiction between our results and those of Bollman, Mann and Magath [1924] who found that the liver is the only organ in the body which produces urea. The growing embryonic kidney can, however, produce ammonia, urea or both together. On the other hand, the presence of a little glucose frequently prevents the appearance of these substances, and in the experiments of Bollman, Mann and Magath the blood-sugar was kept at a normal, even slightly supernormal, level. Thus it is not possible to state at present that the formation of urea occurs only when the kidney tissue is growing, and not in the adult, functioning kidney. The origin of the ammonia, which is excreted by functioning kidney when the glomerular filtrate is acid, is not known.

What has been commonly spoken of as the "protein-sparing" power of carbohydrates is in the intact animal a complex phenomenon. It seems of interest to have demonstrated directly such an action in the case of an isolated tissue removed from the immediate influence of insulin and other hormones which affect the metabolism in the body of the whole animal.

SUMMARY.

(1) Addition of glucose to the medium makes no difference to the nonoccurrence of ammonia and urea production by non-growing, but surviving embryonic rat-kidney tissue.

(2) Addition of glucose to embryonic rat-kidney tissue growing *in vitro* generally inhibits the production of ammonia and urea which would otherwise take place, and frequently actually causes a reduction in the amount of these substances initially present.

We should like to thank Sir F. G. Hopkins for his interest in this research. We are indebted to the Medical Research Council for grants which have made this work possible.

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CLXXXIII. THE EFFECT OF ADRENALINE ON KETOSIS IN PHLORIDZINISED AND NORMAL RATS.

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(Received October 31st, 1927.)

In the course of metabolic experiments on phloridzinised rats it was observed that doses of adrenaline increased the excretion of acetone bodies. This paper gives an account of some experiments undertaken in an attempt to investigate this increase and a discussion of its significance in relation to other published observations on the action of adrenaline.

Rats were phloridzinised by Coolen's method, receiving 50 mg. phloridzin in oil every day or every two days. Urine was collected in 24-hour samples in the usual way. Acetone bodies were estimated by the method used by Wigglesworth [1924]. A satisfactory diet for the fully phloridzinised rat had been found in previous work to consist of caseinogen 75 parts, fat 20, salts 5, agar-agar 5. The salt mixture was that employed by Wigglesworth ($Ca_3(PO_4)_2$ 15, Na_2HPO_4 10, KH_2PO_4 27·5, NaCl 10, KCl 5). On this diet the animals remained in fairly good condition even when completely phloridzinised for periods up to 3 weeks. The daily excretion of acetone bodies was about 4–8 mg. in normal rats, rising slightly on phloridzinisation to 5–15 mg.

The subcutaneous administration of adrenaline in doses of 0·5 mg. to phloridzinised rats caused a large rise in the excretion of acetone bodies. Figs. 1 and 2 show the effect in two animals: in (1) the total acetone bodies rose after administering adrenaline to 63 mg. and in (2) to 62 and 82 mg.; in each case the excretion fell to the normal level next day. In non-phloridzinised rats on the above diet or on a standard diet containing carbohydrates no rise in acetone body excretion was observed after the administration of adrenaline (see Fig. 1, days 1–5, and Fig. 3). In non-phloridzinised rats on a diet of fat with 5 % salts, adrenaline produced a small rise, the effect being more marked in young animals in which there was a greater ketosis. Thus Fig. 4 for an old rat shows a rise from 2·5 mg. acetone bodies to 5·7 mg. after adrenaline, and Fig. 5 for a young animal shows a larger initial ketosis of about 11 mg. gradually falling to 2 mg., with peaks of 23·9, 24·3, and 14·3 mg. after adrenaline. It was found that rats on this fat diet could not survive phloridzinisation for more than a day or two.

In view of the possibility that this rise in ketosis might be due to the acidosis produced by adrenaline, rats on the protein-fat diet + 5 % $NaHCO_3$ were phloridzinised and given adrenaline. The excretion of acetone bodies was at a higher level in these animals, as was expected from the finding of Wigglesworth [1924] that alkali produces ketosis, but adrenaline still caused a large rise comparable with that obtained on the protein-fat diet without alkali. Fig. 6 illustrates this, the rise being from about 20 mg. to 68 mg., falling to the original level next day.

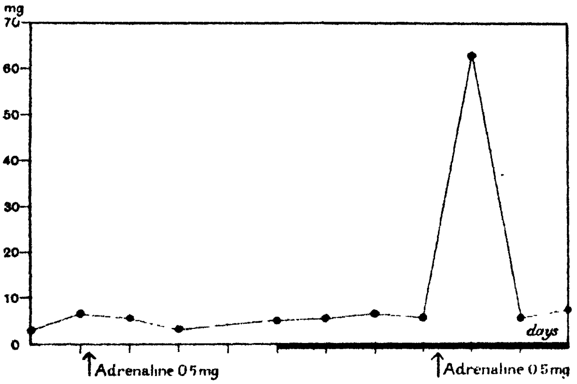

Fig. 1. Abscissa: days. Ordinate: excretion in mg. per day of acetone bodies (acetone + acetoacetic acid + β-hydroxybutyric acid). Rat of about 165 g., put on protein-fat diet 4 days before beginning of experiment. Thickened portion of base line indicates period of phloridzinisation.

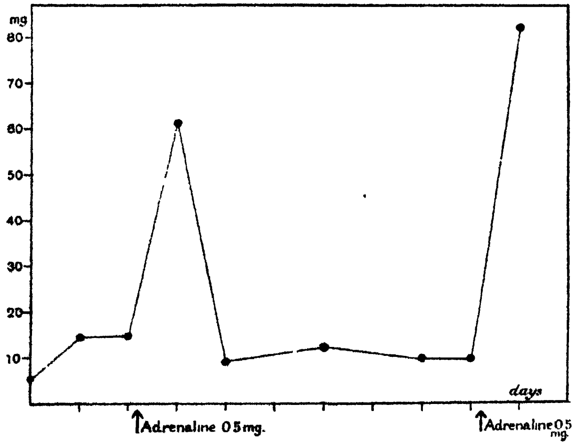

Fig. 2. Abscissa and ordinate as in Fig. 1. Rat of about 200 g., put on protein-fat diet one day before beginning of experiment; phloridzinised throughout experiment.

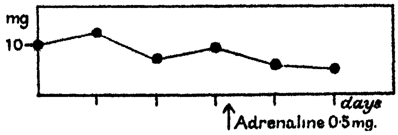

Fig. 3. Abscissa and ordinate as in Fig. 1. Rat of about 190 g., put on standard complete diet 9 days before beginning of experiment.

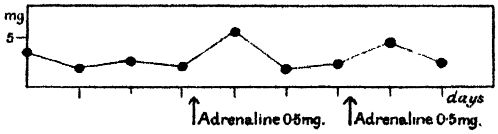

Fig. 4. Abscissa and ordinate as in Fig. 1. Rat of about 195 g., put on fat diet 2 days before beginning of experiment.

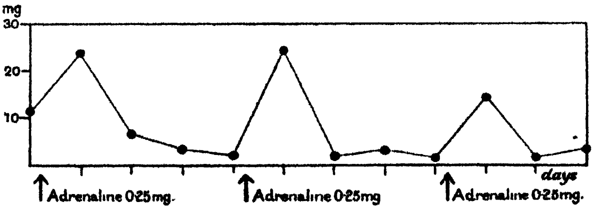

Fig. 5. Abscissa and ordinate as in Fig. 1. Young rat of about 130 g., put on fat diet 3 days before beginning of experiment.

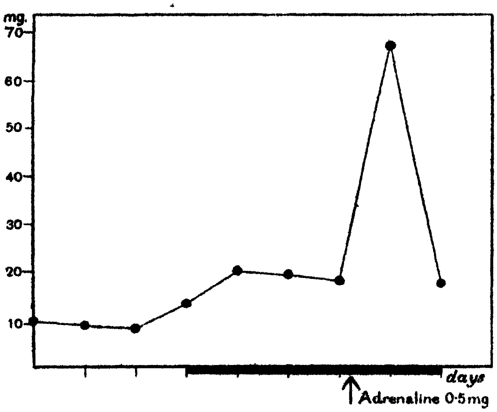

Fig. 6. Abscissa and ordinate as in Fig. 1. Rat of about 195 g., put on protein-fat diet + 5% $NaHCO_3$ 3 days before beginning of experiment. Thickened portion of base-line indicates period of phloridzinisation.

DISCUSSION.

In the experiments reported above we found a large rise in the excretion of acetone bodies with a quick return to the previous level. Since the effects of adrenaline only last for a few hours, this striking increase in the excretion of acetone bodies is no doubt even more intense than our figures for the

24 hour periods show. It seems that adrenaline may have a specific effect on fat metabolism, possibly in hastening β-oxidation and thereby the production of four-carbon residues. The increase in excretion of acetone bodies was only observed in cases where there was already a tendency to ketosis. If adrenaline exerts a specific stimulus to fat metabolism under normal conditions of diet, the amount of acetone bodies produced is not more than can be completely oxidised by the body.

That adrenaline causes a rise in metabolism is a well established fact; heat production and oxygen consumption are both increased. Lusk and Riche [1914], Palladin and Tichwinskaja [1925], and other observers have found no increase in protein metabolism. Increased combustion of carbohydrate might be suspected in view of adrenaline hyperglycaemia. But Boothby and Sandiford [1923] have pointed out that "the calorigenic action of adrenalin cannot be due simply to the presence of excess carbohydrate," for ingestion of sugar resulting in a blood-sugar much in excess of that in adrenaline hyperglycaemia gave only a small rise in heat production; an experiment on a diabetic showed no increase in metabolism after ingestion of 100 g. glucose but a marked increase after adrenaline with only a slight rise in blood-sugar. Stricker and Bouckaert [1924] injected glucose slowly into dogs till the blood-sugar was up to 0·3 % and found a slight fall in heat production, while adrenaline caused a sharp rise for a rather smaller hyperglycaemia. Thus the increase in metabolism after adrenaline cannot be wholly explained by the increase in free glucose.

The rise in the R.Q. after adrenaline found by most workers appears at first sight to rule out the possibility of an increase in fat metabolism. But a careful investigation of the figures given by Boothby and Sandiford [1923] and Krantz and Means [1926] shows that this rise is small and seems to precede the peak of heat production, pulse rate, blood pressure, etc. The apparent rise in R.Q. may be due to a washing out of CO_2 from the blood consequent on the decrease of alkaline reserve caused by adrenaline; thus Bornstein [1921] found that adrenaline caused increased lung ventilation and suggests that the temporary rise in R.Q. is due to loss of CO_2 and not necessarily to a larger combustion of carbohydrate. He observed that after the preliminary rise the R.Q. usually fell below normal.

The experiments of Hubbard and Wright [1921] on men and those of Raab [1926] on dogs may be quoted as more direct evidence that adrenaline affects fat and ketone body metabolism; these workers found an increase in acetone bodies in the blood after the administration of adrenaline, while Raab and also Fleisch [1926] observed a fall in blood-fat.

A temporary acidosis after adrenaline has been observed by Foa and Gatin-Gruzewska [1905], Peters and Geyelin [1917], Elias and Sammartino [1921], and others. In our experiments in which adrenaline was given to phloridzinised rats on a diet containing sufficient $NaHCO_3$ to produce an alkalosis, the rise in acetone bodies after adrenaline was comparable with that obtained without the addition of bicarbonate to the diet. Also Wigglesworth [1924] found that an acidosis caused by NH_4Cl or $NH_4H_2PO_4$ reduced ketosis due to a fat diet or to alkali sometimes to a level below that found in rats under normal conditions. From these considerations it would seem that the acidosis is not responsible for the rise in excretion of acetone bodies after adrenaline.

In a recent paper Levine and Smith [1927] report that fasting for periods of from 4 to 7 days produced no appreciable ketosis in the rat, and that the output of acetone bodies during starvation was similar to that of animals consuming various diets. Thus in our experiments a lack of food intake during the adrenaline period cannot be responsible for the increased ketosis.

We suggest that the large rise in excretion of acetone bodies caused by adrenaline in phloridzinised or fat-fed rats may be due to a temporary intense stimulus to the oxidation of fat, the production of acetone bodies being increased beyond the capacity of the body to oxidise these substances.

SUMMARY.

1. The administration of adrenaline to phloridzinised rats on a protein-fat diet causes a very large temporary increase in the excretion of acetone bodies.

2. In non-phloridzinised rats on the protein-fat diet or on a standard diet containing carbohydrates, no rise in acetone body excretion is observed after the administration of adrenaline.

3. Adrenaline causes an increase in the ketosis of rats on a fat diet.

4. Addition of $NaHCO_3$ to the protein-fat diet does not prevent this effect of adrenaline.

5. It is suggested that the large rise in excretion of acetone bodies caused by adrenaline may be due to a temporary intense stimulus to the oxidation of fat.

We wish to thank Sir F. G. Hopkins and Mr J. B. S. Haldane for their interest in this work, which was done during the tenure by M. D. A. of a Fellowship at Newnham College and a part-time grant from the Medical Research Council.

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Note added December 3rd, 1927. While this paper was in the press, Burn and Ling (*J. Physiol.* **64**, xxii) published work reporting that adrenaline diminishes or inhibits ketonuria in rats on a diet of fat. The conditions were not the same as in our experiments: the doses of adrenaline were smaller and more frequent, and were given during the larger initial ketosis of the 2nd and 3rd days on the fat diet, not as in our experiments, after the animals had adapted themselves to the diet.

CLXXXIV. THE AUTOCATALYTIC OXIDATION OF SULPHYDRYL COMPOUNDS.

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In previous papers by Warburg and Sakuma [1923], by Toda [1926] and by the author [Harrison, 1924, 1927], it was shown that minute traces of certain catalytic metals such as iron are capable of producing a great acceleration in both the aerobic and the anaerobic oxidation of sulphydryl compounds, such as cysteine and glutathione. When this catalytic effect of the metals was prevented, either by careful purification of the sulphydryl compounds from traces of metallic impurities, or by forming a non-catalytic complex of the metals with potassium cyanide, the rate of uptake of oxygen or the rate of reduction of methylene blue by the sulphydryl compounds was found to be very much reduced. It had been claimed by Dixon and Tunnicliffe [1923] that the oxidation of sulphydryl compounds was autocatalytic. By measuring the rate of reduction of methylene blue by sulphydryl compounds, they had obtained the **S**-shaped curves typical of autocatalytic reactions. Further, they had shown that the aerobic and anaerobic oxidation of a sulphydryl compound was accelerated by the addition of the oxidised, *i.e.* disulphide, form, either of the same or of some other sulphydryl compound. When it was found, as indicated above, that the presence of metallic impurities ordinarily present in sulphydryl compounds exerted such a marked effect in determining the velocity of both the aerobic and anaerobic oxidation of the sulphydryl compounds, the possibility suggested itself that the accelerating effect on the aerobic and anaerobic oxidation of the SH group observed by Dixon and Tunnicliffe when a disulphide was added to the solution might in reality be due to the catalytic action of traces of metals such as iron and copper present in the added disulphide, rather than to a catalysis by the disulphide *per se*. The criticism of such a theory that it would not explain the autocatalytic curves obtained by Dixon and Tunnicliffe from measurements of the rate of reduction of methylene blue by sulphydryl compounds, does not necessarily carry much weight, since Dixon and Tunnicliffe, in a note at the end of their paper, stated that the autocatalytic nature of the curve was due partly to an initial retardation of the reaction by traces of oxygen present in the vacuum tubes. This oxygen tended to reoxidise the methylene white and led to a retardation in the initial velocity of the decoloration of the methylene blue. On taking greater precautions to remove traces of oxygen, the curves were found to become more linear, though their autocatalytic nature never completely disappeared. It was suggested by the author [Harrison, 1924] and by Oppenheimer [1926] that traces of iron impurities might be playing a part in the catalytic effects observed by Dixon and Tunnicliffe. When, on studying the catalytic action of metals on the oxidation of the SH group, it was found that a marked acceleration was produced by concentrations of iron and copper of the order of 10^{-5} mg. per cc., or even less, it seemed worth while to attempt to find whether the catalysis observed by Dixon and Tunnicliffe was a catalysis by the disulphide itself, or was due to the catalytic action of metallic impurities present in the disulphide compound.

The following experiments suggested themselves as means of deciding this question.

(*a*) To prepare a pure disulphide compound taking every possible precaution to remove traces of metallic impurities, and to try whether this purified compound were still capable of catalysing the oxidation of sulphydryl compounds.

(*b*) To find whether the catalysis were affected by the addition of cyanides, which form non-catalytic complexes with catalytic metals.

(*c*) To destroy the —S—S— linkage by acting on the carefully purified disulphide with a suitable reagent, and then to test whether the resulting product still possessed catalytic properties. If, after this treatment, the catalysis were still produced, it would be evident that it could not be a property of the disulphide group.

EXPERIMENTAL.

The preparation of metal-free dithiodiglycollic acid.

Dithiodiglycollic acid was chosen as the most suitable disulphide, since it could be prepared by oxidising thioglycollic acid with iodine [Biilmann, 1905] and both the thioglycollic acid and the iodine could be obtained metal-free by distillation in quartz vessels. The iodine was purified by distillation from quartz in a current of steam. (This method was used in preference to the sublimation method of Toda [1926], since the risk of traces of metallic halides distilling over in the iodine vapour was minimised.) All the solvents used in the preparation were first distilled from quartz. 7·3 g. of thioglycollic acid (previously distilled 3 times from quartz at 16 mm. Hg pressure) was dissolved in 10 parts of pure water, and pure iodine was slowly added with shaking until a very slight excess was present. The solution was shaken with 3 volumes of ether, the ether layer being then carefully poured off and shaken with a little water. After standing, the ether layer was again poured off, the ether was evaporated and the residue dried at 100° in a quartz dish until nearly all the excess of iodine had been removed. The residue was dissolved in the minimum quantity of hot ethyl acetate, treated with 9 volumes of benzene and allowed to crystallise. After being washed by decantation three times with a mixture of ethyl acetate and benzene, the crystals were dissolved in hot ethyl acetate and the solution poured into a quartz dish, the solvent being evaporated in a vacuum desiccator. 2·9 g. of perfectly colourless, crystalline dithiodiglycollic acid were obtained, melting at 106–107°.

The catalytic action of metal-free dithiodiglycollic acid.

Experiments were then carried out to find whether the purified dithiodiglycollic acid behaved like the unpurified disulphides in being capable of accelerating the reduction of methylene blue by sulphydryl compounds. The glass vacuum tubes employed in these experiments were well cleaned each time before use in the manner previously described [Harrison, 1927]. In all the experiments on the reduction of methylene blue, 0·3 cc. of a 1 in 5000 aqueous solution of the dye was used in each vacuum tube. The dithiodiglycollic acid was dissolved in pure water and neutralised with dilute ammonium hydroxide, previously distilled in quartz vessels. The alkali was added from a quartz burette, the amount required being determined by a separate titration. The required volume of neutralised dithiodiglycollic acid was added from the quartz burette to a mixture of 0·3 cc. of 1 in 5000 methylene blue solution, 0·1 cc. of neutralised sulphydryl compound (cysteine, thioglycollic acid or glutathione), and sufficient phosphate buffer of the required p_H to make the total volume of solution in each tube equal to 2·0 cc. In the control tubes, instead of the solution of dithiodiglycollic acid, an equal volume of pure water was added. In many of the experiments employing dithiodiglycollic acid, the latter was neutralised with sodium hydroxide instead of ammonia, since it was found that by using the former reagent the solution could be more easily maintained at the required p_H. The sodium hydroxide used was a very pure specimen made from sodium, and in every experiment the same amount of alkali, neutralised with quartz-distilled hydrochloric acid, was added to the control tube. A second control tube was used in which pure water replaced the neutralised alkali. In every case the two controls became decolorised at the same time, showing that the sodium hydroxide used to neutralise the disulphide did not contain impurities which in any way affected the rate of reaction. No special precautions were taken to obtain the sulphydryl compounds, methylene blue or phosphate buffer free from the last traces of metal impurities, since the same amount of these substances was added to both the experimental and the control tubes. The tubes were all evacuated together by means of the water-pump and filled with nitrogen (washed with alkaline pyrogallol). This was repeated and the tubes were finally evacuated completely and immersed in a water-bath maintained at the required temperature. The p_H was checked at the end of each experiment by introducing a few drops of the appropriate indicator through the side tube of the vacuum tube. The tubes in the bath were illuminated as uniformly as possible and protected from direct sunlight, since the latter appears to accelerate the reaction. The experiments were carried out in duplicate, and the reduction times of duplicate tubes showed good agreement.

Table I gives a comparison of the time required for the reduction of methylene blue by thioglycollic acid and glutathione in the absence and in the presence of metal-free dithiodiglycollic acid (R.S.S.R.).

Table I.

R.S.S.R. = metal-free dithiodiglycollic acid. Temp. = 37°.

No.	Sulphydryl compound	p_H	Time in minutes for decoloration of methylene blue	
1	Thioglycollic acid (1·2 mg.)	7·4	Without disulphide	145
	,, ,,	,,	+4 mg. R.S.S.R.	50
2	Thioglycollic acid (1·2 mg.)	7·6	Without disulphide	110
	,, ,,	,,	+4 mg. R.S.S.R.	45
3	Thioglycollic acid (1·6 mg.)	7·6	Without disulphide	63
	,, ,,	,,	+4 mg. R.S.S.R.	23
4	*Thioglycollic acid (2·4 mg.)	7·4	Without disulphide	280
	,, ,,	,,	+4 mg. R.S.S.R.	27
	,, ,,	,,	+8 mg. R.S.S.R.	10
5	Glutathione (1·2 mg.)	7·6	Without disulphide	53
	,, ,,	,,	+4 mg. R.S.S.R.	29

* Freshly distilled thioglycollic acid.

The experimental results given in Table I are typical of a large number which have been obtained. It will be observed that a large acceleration in the rate of reduction of methylene blue by the sulphydryl compounds is produced by the addition of the purified dithiodiglycollic acid. What appears still more significant is the fact that the acceleration produced by a given amount of the metal-free disulphide is of the same order as that recorded by Dixon and Tunnicliffe using the unpurified disulphide. If the acceleration produced by the purified disulphide were due to minute traces of catalytic metals which had escaped removal, it would have been expected that the catalysis observed on adding the purified disulphide would have been of a much lower order than that produced by the unpurified, metal-containing disulphide. The fact that no appreciable reduction in catalytic power occurs, even after very careful purification, would appear to afford strong evidence that the catalysis observed is a property of the disulphide itself. The fact that the metallic impurities in the disulphide used by Dixon and Tunnicliffe caused no appreciable additional catalytic effect is probably due to the quantity of catalytic impurities already present in their unpurified —SH compounds. Still stronger evidence on these lines is given in Exp. 4 in Table I, where, using a freshly distilled specimen of thioglycollic acid with a relatively slow reducing power, the relative acceleration produced by the purified dithiodiglycollic acid was very much greater than that observed by Dixon and Tunnicliffe, using unpurified compounds. The relatively slow rate of reduction of methylene blue by the freshly distilled thioglycollic acid alone is probably due not only to removal of catalytic metals, but also to the removal, by distillation, of any traces of disulphide which might be present owing to slow oxidation of the thioglycollic acid. This would explain the relatively greater effect produced by added disulphide. Two different preparations of dithiodiglycollic acid were made by the method given above, and both accelerated to a similar extent. This is in itself evidence that the catalysis is not due to impurities, since it is unlikely that two different preparations of the disulphide would contain the same amount of impurity.

Table II shows the effect of addition of pure dithiodiglycollic acid to sulphydryl compounds in presence of cyanide. In experiments employing potassium cyanide, a fresh solution of the latter was made up every day, and neutralised with HCl immediately before use.

Table II.

R.S.S.R. = metal-free dithiodiglycollic acid.

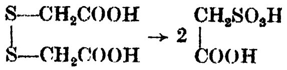
No.	Sulphydryl compound	p_H	Temp.	KCN	Time in minutes for decoloration of methylene blue	
1	Thioglycollic acid (1·2 mg.)	7·6	37°	$M/500$	Without disulphide	102
	" "	"	"	"	+4 mg. R.S.S.R.	62
2	*Thioglycollic acid (2·4 mg.)	7·4	37°	$M/1000$	Without disulphide	410
	" "	"	"	"	+8 mg. R.S.S.R.	17
3	*Thioglycollic acid (2·4 mg.)	7·4	37°	$M/500$	Without disulphide	420
	" "	"	"	"	+4 mg. R.S.S.R.	35
4	Cysteine (0·8 mg.)	7·6	25°	$M/400$	Without disulphide	110
	" "	"	"	"	+4 mg. R.S.S.R.	65
5	Glutathione (1·2 mg.)	7·6	25°	$M/500$	Without disulphide	173
	" "	"	"	"	+4 mg. R.S.S.R.	46

* Freshly distilled.

It is evident from Table II that the acceleration produced by pure dithiodiglycollic acid can still take place even in the presence of cyanide. Moreover, using freshly distilled thioglycollic acid as the —SH compound, the acceleration produced by the disulphide is relatively greater in the presence of cyanide than in its absence. This again affords strong evidence that the acceleration by dithiodiglycollic acid is a true property of the disulphide group itself. Potassium cyanide is well known to form non-catalytic complexes with catalytic metals such as iron and copper, and, if the observed acceleration were due to minute traces of metallic impurities which had escaped removal during purification of the disulphide, one would expect the addition of cyanide to prevent such an acceleration.

The effect of destroying the —S—S— group by bromine.

An attempt was now made to destroy the —S—S— group in the pure dithiodiglycollic acid, which, as has been shown above, is capable of catalysing the oxidation of the sulphydryl group. If, after destroying the disulphide group, the resulting product no longer catalysed the reduction of methylene blue by sulphydryl compounds, additional strong evidence would be afforded that the catalysis is a property of the disulphide group itself. Bromine was chosen as the reagent for destroying the —S—S— group in dithiodiglycollic acid by oxidation. Preliminary experiments showed that an aqueous solution of dithiodiglycollic acid readily reacts with bromine in the cold, the amount of bromine required corresponding with the formation of sulphoacetic acid.



$$\begin{array}{l} S-CH_2COOH \\ | \\ S-CH_2COOH \end{array} \rightarrow 2 \begin{array}{l} CH_2SO_3H \\ | \\ COOH \end{array}$$

This is analogous to the oxidation by bromine of cystine to cysteic acid. The product no longer gave a nitroprusside reaction after treatment with zinc dust and HCl, showing that all the disulphide had been destroyed.

A dilute aqueous solution of pure dithiodiglycollic acid containing 4 mg. per cc. was treated with a slight excess of quartz-distilled bromine, the product being warmed to 110° in a quartz dish till the HBr was removed. The product was then neutralised with pure NaOH, water being added until 1 cc. of the solution contained the oxidised product from 4 mg. of dithiodiglycollic acid. The effect of 1 cc. of this solution (in which the —S—S— linkage has been destroyed by bromine) on the rate of reduction of methylene blue by thioglycollic acid was then compared with (*a*) the rate of reduction of methylene blue by thioglycollic acid alone; and with (*b*) the rate of reduction of methylene blue by thioglycollic acid in presence of 4 mg. of dithiodiglycollic acid (not treated with bromine).

The following results were obtained.

Time for reduction of methylene blue by thioglycollic acid (1·2 mg.)

$p_H = 7·4$ Temp. = 37°

(*a*)	Alone	143 min.
(*b*)	4 mg. dithiodiglycollic acid (—S—S— linkage intact) ...	59 ,,
(*c*)	Product of 4 mg. of dithiodiglycollic acid treated with bromine (—S—S— linkage destroyed)	145 ,,

These results show that after destroying the disulphide linkage in dithiodiglycollic acid with bromine, the product has no power whatever to catalyse the anaerobic oxidation of the sulphydryl group. Had the catalysis been due to metallic impurities, these would have remained in the solution after treatment with bromine, and the catalytic power of the solution would not have been destroyed. The fact, that after destruction of the —S—S— group by bromine the catalysis is no longer manifested, clearly indicates that the catalysis is a function of the disulphide group itself.

Table III shows the effect of increasing concentrations of pure dithiodiglycollic acid on the rate of reduction of methylene blue by freshly distilled thioglycollic acid.

Table III.

Temp. = 37° $p_H = 7·9$

Added dithiodiglycollic acid (mg.)	Time for decoloration of methylene blue by thioglycollic acid (2·4 mg.), min.
—	275
0·4	150
1·0	80
2·0	30
4·0	27

It is evident that marked acceleration is produced in the rate of anaerobic oxidation of thioglycollic acid by relatively small concentrations of pure dithiodiglycollic acid. The fact that 4 mg. of the disulphide produces very little more acceleration than 2 mg. is probably explained by the effects of mass action, the catalytic action of the disulphide being opposed by the tendency of the latter to drive the reaction in the opposite direction. A similar effect was observed by Dixon and Tunnicliffe.

Table IV shows the catalytic effect of pure dithiodiglycollic acid at different hydrogen ion concentrations.

Table IV.

Temp. = 37°

p_H	Dithiodiglycollic acid (mg.)	Time for decoloration of methylene blue by thioglycollic acid (2·4 mg.), min.
6·0	—	255
,,	4	125
6·8	—	255
,,	4	45
7·4	—	150
,,	4	20

The results show that as the solution becomes more acid there is a falling off of the relative acceleration produced by the disulphide. Dixon and Tunnicliffe also mention this.

The catalysis produced by dithiodiglycollic acid cannot be attributed to an appreciable increase in the concentration in the —SH group, brought about by hydrolysis at the —S—S— linkage in the weakly alkaline solutions, since it was found that dithiodiglycollic acid alone showed no ability to reduce methylene blue even on prolonged standing in the bath at 37°. Further, in some of the experiments, using tubes with and without added disulphide, the tubes were opened, 0·1 cc. of concentrated HCl was quickly added to each (to prevent further oxidation), and the solutions were titrated with $N/100$ iodine, using starch as an indicator. The tubes containing added disulphide were found to reduce practically the same amount of iodine as those containing no added disulphide. This shows that any hydrolysis of the disulphide which occurs is of so slight an extent that the amount of —SH formed, in comparison with that already present, is too small to be detected by the above experiment. Even in the presence of KCN, which is known to increase the hydrolysis of the —S—S— group, no decoloration of methylene blue was observed. (5·2 mg. dithiodiglycollic acid, $M/200$ KCN, methylene blue and phosphate buffer of p_H 8·0.)

Ample justification for undertaking the work described in this paper is afforded by the fact that ordinary specimens of so-called pure substances chosen at random, differing as widely as succinic acid and ammonium chloride, were found to be capable of causing an acceleration in the anaerobic oxidation of thioglycollic acid of a similar order to that produced by an equal weight of dithiodiglycollic acid. After subliming the succinic acid as succinic anhydride into a quartz dish and neutralising with pure sodium hydroxide, it was found to be no longer capable of producing any acceleration in the reaction.

The acceleration produced by ammonium chloride did not disappear after sublimation into quartz vessels, but a specimen prepared by neutralising quartz-distilled hydrochloric acid with quartz-distilled ammonia did not produce any acceleration in the reduction of methylene blue by thioglycollic acid. (Probably the failure to destroy the catalytic power of ammonium chloride by sublimation was due to the simultaneous sublimation of the halides of catalytic metals in the atmosphere of dry HCl produced by the dissociation of the ammonium chloride.) It is not surprising that so-called pure substances should be capable of producing such an acceleration in the oxidation of sulphydryl compounds, when one considers the powerful catalytic effect produced on the reaction by extraordinarily minute traces of such metals as copper and iron.

The catalytic action of metal-free dithiodiglycollic acid on the aerobic oxidation of sulphydryl compounds.

Experiments were carried out to find whether the metal-free dithiodiglycollic acid was capable of catalysing the oxidation by oxygen of thioglycollic acid and cysteine. The oxygen uptake was measured by means of the Barcroft microrespirometer, the apparatus being shaken in a water-bath at 37°. The bottles of the apparatus were made of quartz, and were thoroughly cleaned with acids before use. The total volume of liquid in each bottle was 3·0 cc., this volume being made up with phosphate buffer. The dithiodiglycollic acid was neutralised with pure NaOH, an equal amount of the alkali, neutralised with quartz-distilled HCl, being used in the control apparatus.

Fig. 1 shows the catalytic effect produced on the aerobic oxidation of cysteine by the addition of pure dithiodiglycollic acid. It is evident that a large acceleration is produced by the addition of the pure disulphide, and, further, by comparing curves *B* and *C*, it can be seen that an acceleration is brought about by the disulphide even in presence of *M*/900 cyanide. It is very significant that curves *A* and *B* show distinctly the **S**-shaped, sinuous form characteristic of an autocatalytic reaction. Dixon and Tunnicliffe failed to obtain any such evidence of the autocatalytic nature of the reaction in any of their experiments on the aerobic oxidation of sulphydryl compounds. In the case of cysteine, they explain this by the fact that the disulphide formed, namely, cystine, is insoluble, and, being precipitated from the solution, is unable to catalyse the oxidation of the remaining cysteine. In the present experiment, such a precipitation of cystine was observed during the oxidation of the control, containing no added disulphide (Curve *C*), but it was interesting to find that in the two solutions containing added disulphide (Curves *A* and *B*), no precipitation of cystine could be observed, even at the end of the reaction. This ability of dithiodiglycollic acid to prevent the precipitation of cystine is probably due to a mutual interaction of the two disulphides, cystine and dithiodiglycollic acid, of the nature:

$$\mathrm{R.S.S.R.} + \mathrm{R'.S.S.R'.} \rightarrow 2\,\mathrm{R.S.S.R'.},$$

leading to the formation of a soluble product. Hence, during the reaction, the concentration of soluble disulphide is continuously increasing, and one observes not only the catalytic effect of added dithiodiglycollic acid, but also the autocatalytic effect of the increasing concentration of cystine formed during the reaction and held in solution by the dithiodiglycollic acid. The fact that such an autocatalytic curve has now been obtained for the *aerobic* oxidation of a sulphydryl compound affords almost conclusive evidence that the oxidation of the sulphydryl group is catalysed by disulphides.

The control curve *C* appears to be very slightly autocatalytic in shape. This may be due to non-precipitation of cystine in the earlier stages of the reaction, owing to slight solubility or to supersaturation of the solution (such a retention in solution of small quantities of cystine has been observed in the methylene blue experiments, in which no precipitation of cystine could be observed at the end of the reaction); or it might possibly be due to removal of a small amount of cyanide by distillation into the upper part of the bottle, or even by coupled oxidation or decomposition during the reaction. This might allow a slight increase in the activity of traces of catalytic metals present in the cysteine. A similar effect might account for a very small part of the autocatalysis exhibited by curve *B*, but the autocatalytic nature of curve *A* must be due entirely to a catalysis by disulphide since cyanide was not present.

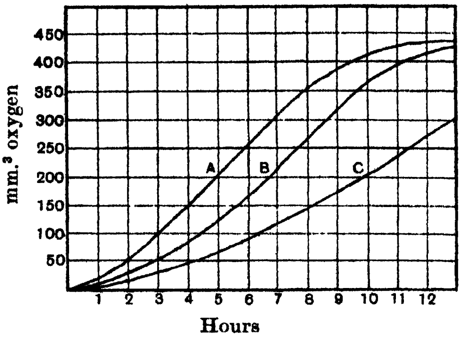


Fig. 1. A. 8 mg. cysteine + 16 mg. pure dithiodiglycollic acid
B. „ „ + „ „ „ „ + $M/900$ KCN
C. „ „ + $M/900$ KCN
$p_H = 7·4$.

It may be mentioned that in an unpublished experiment carried out by the author in 1924 it was found that the addition of 45 mg. of oxidised glutathione (freed as completely as possible from catalytic metals by the method described [Harrison, 1924]) to 13·5 mg. of pure cysteine hydrochloride, brought about a marked acceleration in the rate of oxidation of the latter (temp. = 37°; $p_H = 7·6$; vol. = 3 cc.). Here again it was observed that no precipitation of cystine occurred in the bottles containing added oxidised glutathione, though in this case, owing to the rapid oxygen uptake, the readings taken were not sufficiently accurate to determine whether or not the reaction was autocatalytic.

It has been found that the aerobic oxidation of thioglycollic acid is also catalysed by the addition of pure dithiodiglycollic acid. 8 mg. of thioglycollic acid in 3 cc. of water (temp. = 37°, $p_H = 7{\cdot}4$), took up 13·7 mm.^{3} of oxygen in the first 11 hours, while an equal amount of thioglycollic acid to which 16 mg. of pure dithiodiglycollic acid had been added took up 72·4 mm.^{3} of oxygen over the same period of time.

DISCUSSION.

It has been shown by the experiments described that the catalytic effect produced in both the aerobic and anaerobic oxidation of sulphydryl compounds by the addition of disulphides is not due to catalytic impurities present in the disulphide, but is a true property of the disulphide itself. The question arises as to explanation of this catalytic effect. Dixon and Tunnicliffe advance the hypothesis that an addition compound between the sulphydryl compound and the disulphide is formed, and that the compound oxidises more rapidly than the sulphydryl compound itself. The evidence they give in support of this hypothesis is very slender, and definite reasons can be given against the likelihood of the formation of such an addition compound. Sidgwick [1927] has recently pointed out that certain substances, such as water and the alcohols, show many abnormal physical properties, owing to the formation of complexes by molecular association. When the oxygen in these compounds is replaced by sulphur, the resulting compounds show normal physical properties, indicating that association has not taken place. Unlike oxygen compounds, sulphur compounds do not show a tendency to complex formation.

It seems possible that the catalytic effect of the disulphide compounds on the oxidation of the sulphydryl compounds may be explained by the following mechanism. Firstly, the disulphide, when in solution, is, to a very slight extent hydrolysed to a mixture of the corresponding sulphydryl compound and the sulphenic acid:

$$(1)\quad \text{R.S.S.R.} + \text{H}_2\text{O} \rightleftharpoons \text{R.SH} + \text{R.SOH}.$$

Secondly, the sulphenic acid takes up oxygen to form the sulphinic acid:

$$(2)\quad \text{R.SOH} + \text{O} \rightleftharpoons \text{R.SOOH}.$$

Thirdly, if a sulphydryl compound (R′SH) is present, it reduces the sulphinic acid:

$$(3)\quad 2\text{R}'\text{SH} + \text{R.SOOH} \rightleftharpoons \text{R}'\text{.S.S.R}'. + \text{R.SOH}.$$

The sulphydryl compound thus becomes oxidised to the disulphide, and the sulphenic acid is regenerated. If the oxidation of the sulphenic acid to the sulphinic acid, and the re-reduction of the latter by the sulphydryl compound, together take place more rapidly than the direct oxidation of the sulphydryl compound by the air (and experiments have shown that the latter reaction in the absence of metals is very slow), then a positive catalysis of the oxidation will be expected on the addition of disulphide.

That such reactions as those suggested above can actually take place appears likely from the following considerations. Reaction (1), namely, the hydrolysis of the —S—S— group to a mixture of —SH and —SOH is given by Smiles [1911] as the way in which the breakdown of aromatic disulphides in acid or alkaline solution takes place. Evidence is given by Smiles and Stewart [1921] that in the case of the hydrolysis of *m*-dithiobenzoic acid (which takes place on treatment with cold aqueous NaOH) the primary products are the sulphydryl compound and the sulphenic acid.

The isolation of the sulphenic acids has not apparently been achieved so far, except in the case of anthraquinonesulphenic acid [Fries, 1912], but derivatives can be isolated. Smiles points out that the hydrolysis (1) is reversible and that the equilibrium is greatly in favour of the disulphide. As regards reaction (2), it has been shown that anthraquinonesulphenic acid in solution readily takes up oxygen from the air to form the corresponding sulphinic acid. (Indeed the occurrence of sulphinic acid in alkaline solutions of a disulphide [Fromm and Wittmann, 1908] was one of the reasons for suggesting the existence of sulphenic acids.) There is little doubt that reaction (3), namely, the reduction of the sulphinic acid by the sulphydryl compound can take place, for Otto [1888] has shown that phenylsulphinic acid is reduced by hydrogen sulphide, and Gilman, Smith and Parker [1925] find that even toluenesulphonyl chloride is reduced by R.SH. Many of the aliphatic sulphinic acids have been isolated, and the fact that the existence of the aliphatic sulphenic acids is probably only transitory does not in any way mean that they are necessarily incapable of taking part in such a catalysis as that pictured above.

In the case of the anaerobic oxidation of sulphydryl compounds in which methylene blue replaces oxygen, the catalysis by disulphides may be assumed to take place on exactly similar lines, the reversible reaction

$$(2\,a)\quad \mathrm{R.SOH} + \mathrm{Mb} + \mathrm{H_2O} \rightleftarrows \mathrm{R.SOOH} + \mathrm{Mb.H_2}$$

being substituted for reaction (2). Reaction (2 *a*) will proceed in a forward direction leading to complete reduction of the methylene blue, provided that the sulphinic acid R.SOOH is continuously removed by re-reduction by the sulphydryl compound.

It is not essential that the actual compounds concerned in the intermediate reactions should necessarily be those mentioned. It is merely suggested that the formation of compounds of such a type may be responsible for the catalysis by disulphides.

If the catalysis by disulphides takes place by the mechanism described, in which the first stage involves hydrolysis of the disulphide, it might be expected, since this hydrolysis takes place to a greater extent in alkaline solution, that increasing the p_H of the solution would bring about an increase in the catalytic power of the disulphide. That such is actually the case is evident from the experiment shown in Table IV. This increase of catalysis in alkaline solution thus affords some evidence for the theory suggested.

Previously quoted experiments indicate that near neutrality, if hydrolysis of the —S—S— group occurs, it does so to a very small extent. In other words, reactions (1) and (2) proceed in a forward direction only very slightly. This does not in any way preclude the possibility that hydrolysis may occur to a sufficient extent to bring about the catalysis.

Considering the experiments as a whole, there seems no doubt that the catalytic effect of disulphides on the oxidation of sulphydryl compounds is a true property of the —S—S— group and is not due to metal impurities.

SUMMARY.

(1) Both the aerobic and anaerobic oxidations of sulphydryl compounds are catalysed by pure (metal-free) dithiodiglycollic acid.

(2) This catalysis is not inhibited by cyanide.

(3) By destroying the —S—S— linkage in pure dithiodiglycollic acid, its catalytic action disappears.

(4) A mechanism is suggested by which the catalysis may take place.

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CLXXXV. THE USE OF THE STARCH-IODINE END-POINT IN TUNNICLIFFE'S METHOD FOR THE DETERMINATION OF GLUTATHIONE IN TISSUES.

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(*Received November 7th, 1927.*)

IN the first publication of his admirably simple method for the estimation of glutathione in biological material Tunnicliffe [1925] observed that if it were attempted to carry out the iodine titration of the trichloroacetic acid filtrate with starch as an internal indicator, figures were obtained which were substantially higher than those yielded with nitroprusside used as an external indicator. This finding was later corroborated by Voegtlin and Thompson [1926] and more recently by Kennaway and Hieger [1927]. On the other hand, Uyei [1926] claims to have obtained correct figures with starch as an internal indicator, giving analyses for labile sulphur in support of his claim.

In considering the possible causes for this discrepancy between the nitroprusside and starch titration figures it occurred to the writers that one of the indispensable conditions for the achievement of a good starch-iodine end-point was lacking in the procedure as given by Tunnicliffe. It will be recalled that the titration is carried out with very dilute, $N/100$, or even more dilute, iodine solutions. Such solutions are usually prepared by dilution from $N/10$ stock iodine solutions which contain a certain amount, usually 25 g. per litre, of potassium iodide. Therefore, in the titration of the glutathione with $N/100$, or with more dilute, iodine solutions practically no potassium iodide is present. That the presence of a considerable excess of iodide in acid solution is necessary for the production of a relatively permanent blue starch-iodine compound is an old and well-established fact in analytical chemistry.

In applying this principle to the determination of glutathione by Tunnicliffe's method in various tissue extracts it was found that a strikingly close correspondence between the nitroprusside and starch figures could be obtained, as demonstrated in Table I.

The modification simply consists in adding to the trichloroacetic acid extract to be titrated about 2 cc. of a 25 % solution of potassium iodide

(0·5 g.). Since the disappearance of the blue colour from a solution containing an excess of iodine on the addition of thiosulphate appears to be an even more sensitive reaction than the appearance of the blue colour with iodine, it was found advisable to add a known excess of the iodine solution and to titrate back with an equivalent thiosulphate solution.

The advantages of using the extremely delicate starch-iodine end-point, and of an internal rather than an external indicator, are obvious and require no further comment, beyond drawing attention to our observation, as shown in Table I, that much smaller quantities of glutathione can be determined by means of titration with 0·001 *N* iodine and thiosulphate solutions than could possibly be accomplished with the nitroprusside end-point, which is extremely unsatisfactory for small concentrations of glutathione.

Table I.

Trichloroacetic acid extract		Nitroprusside end-point		Starch end-point without KI		Modified titration with $Na_2S_2O_3$ and added KI, starch end-point	
Tissue	Amount used cc.	cc.	I solution normality	cc.	I solution normality	cc.	I solution normality
Rabbit liver	5·0	2·10	0·005	2·37	0·005	2·05	0·005
Rabbit liver	5·0	3·09	0·005	3·32	0·005	3·04	0·005
	5·0	3·08	,,	3·37	,,	3·07	,,
Rabbit liver	10·0	5·12	0·005	5·80	0·005	5·21	0·005
Mackerel liver	6·0	1·19	0·004	1·31	0·004	1·16	0·004
	3·0	—	—	—	—	0·58	,,
Mackerel liver	5·0	0·92	0·005	1·28	0·005	0·96	0·005
	5·0	0·90	,,	1·23	,,	0·91	,,
	5·0	0·90	,,	1·25	,,	0·91	,,
	10·0	(1·82 calc'd	,,)	2·21	,,	1·79	,,
Mackerel liver	10·0	0·27	0·004	0·59	0·004	0·27	0·004
	5·0	(0·54 calc'd	0·001)	1·11	0·001	0·56	0·001
	10·0	(1·08 ,,	,,)	1·83	,,	1·15	,,
Dogfish liver	15·0	1·47	0·005	1·85	0·005	1·54	0·005
	15·0	1·50	,,	1·87	,,	1·50	,,
Human red blood cells	10·0	1·21	0·002	1·48	0·002	1·20	0·002
Yeast	5·0	3·40	0·002	4·10	0·002	3·45	0·002
	5·0	3·44	,,	4·16	,,	3·42	,,

Although the proposed modification offers a more convenient and perhaps more accurate analytical procedure, the possibility of the presence in tissue extracts of substances other than glutathione which are capable of combining with iodine must not be overlooked. It is certainly advisable in any series of determinations to make a preliminary comparison of the KI-starch-iodine titration with the nitroprusside-iodine method, employing preferably quite concentrated glutathione solutions, to make certain that no such substances are encountered. In a rather extensive series of determinations in trichloroacetic acid extracts of animal tissues the present writers have not encountered any such discrepancies, with the single exception of bile-containing liver extracts, in which case the starch-iodine method yielded somewhat higher figures.

SUMMARY.

The addition of potassium iodide to trichloroacetic acid extracts of tissues permits the employment of the delicate starch-iodine end-point in the titration of glutathione by Tunnicliffe's method.

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CLXXXVI. A NOTE ON THE HYPOGLYCAEMIC ACTION OF DIHYDROXYACETONE IN MAN.

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(*Received November 7th, 1927.*)

SEVERAL interesting and apparently conflicting observations have accumulated about dihydroxyacetone.

(I) When administered to dogs [Campbell and Soskin; Himwich, Rose and Malev, 1926] or human beings [Cathcart and Markowitz, 1927], it causes a much prompter rise in the R.Q. to unity or over than does an equivalent quantity of glucose.

(II) It augments considerably the hyperglycaemia of depancreatised dogs, by whom it is quantitatively excreted as glucose. During this process the R.Q. shows a slight fall [Campbell and Markowitz, 1927].

(III) It exerts no curative influence on the hypoglycaemia following hepatectomy [Markowitz and Campbell, 1927].

(IV) When injected into eviscerated nephrectomised preparations, it enters unchanged into the tissues, the concentration of this substance in the tissues, at the end of several hours (as calculated from the concentration of the triose in a muscle), being sufficient to account for most of the triose administered [Markowitz and Campbell, 1927].

(V) It has no influence on the respiratory metabolism of excised amphibian muscle [Meyerhof, Lohman and Meier, 1925].

Observation (I) suggests that the triose is burnt with much greater facility than glucose. However, observations (II)–(V), if taken in conjunction, make it reasonably certain that dihydroxyacetone cannot be used as such, but must first be converted into glucose by the liver. The explanation for the high R.Q. following the administration of the triose is somewhat conjectural, but (as stated in our previous communication [Cathcart and Markowitz, 1927]) we believe it to depend on the following consideration. A depancreatised dog of only about 6 kg. can excrete nearly 20 g. of dihydroxyacetone as glucose in less than 6 hours. It is therefore a fair assumption that, following the administration of the triose to the intact animal, the tension of carbohydrate in the liver is considerably increased. Presumably the increase is sufficient not only to stop glyco-neogenesis out of fat and protein, but also to bring about a considerable synthesis of fat.

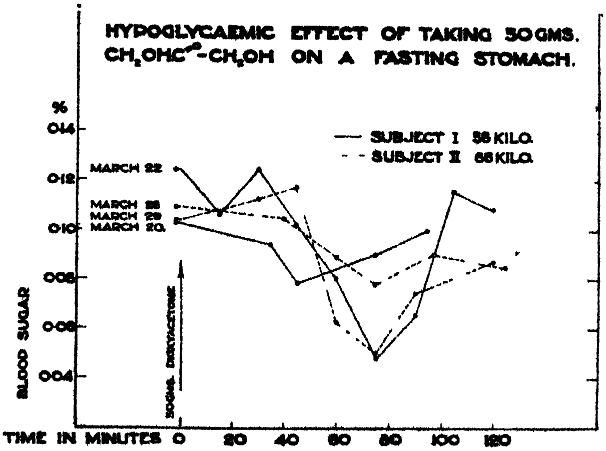
In agreement with the finding that dihydroxyacetone is a sugar-former in the animal body is the observation that it can act as an antidote to the hypoglycaemia following overdosage with insulin both in man and the lower animals [Campbell and Hepburn, 1926; Kermack, Lambie and Slater, 1926]. In decided contrast to this latter property of dihydroxyacetone is the observation, first made by Isaac and Adler [1924] and confirmed by Rabinowitch [1925], that the triose has a hypoglycaemic action in the human subject.

In the course of an investigation which had quite a different object, we found that, following the ingestion of 50 g. dihydroxyacetone on a *fasting* stomach, the subject developed muscular tremor, flushing, restlessness and general malaise. The condition was not recognised until routine blood-sugar determinations, which were made during the course of the experiments, revealed that the symptoms coincided with the existence of hypoglycaemia.

Since dihydroxyacetone thus resembles glucose in that it can both relieve insulin hypoglycaemia and definitely induce hypoglycaemia in man, it was deemed necessary to confirm its blood-sugar-lowering properties.

METHODS.

Five experiments in all were done on two subjects. One of these experiments was reported in Fig. 5 of our previous communication [1927]. The results of the remaining four experiments are presented in the form of a chart. Blood-sugars were estimated by MacLean's method. No correction for the presence of dihydroxyacetone in the first few samples has been introduced. Mason [1926], Campbell (personal communication) and others have shown that the quantity appearing in the blood after such doses is insignificant.



DISCUSSION.

It requires to be explained why a substance which forms sugar so readily in the animal body should have a hypoglycaemic action. The possible mechanism of this action can as yet be only conjectured.

The sugar of the blood is normally a balance between (1) the production of sugar by the liver [Soskin, 1927], (2) the utilisation or storage of sugar by the tissues, and (3) the secretion of insulin by the pancreas. A fall in the blood-sugar must be due to one or more of these factors, each of which will be discussed in turn.

(1) Although there is a considerable rise in the oxygen consumption immediately following the administration of dihydroxyacetone, this is by no means comparable to that occurring even in mild muscular exercise, in which condition there is no hypoglycaemia. It is, therefore, justifiable to conclude that the hypoglycaemia is not due to the increased energy requirement of the organism, although this latter may be a contributory cause.

(2) Before one can state that the hypoglycaemia of dihydroxyacetone is not insular in origin, it must be shown that when administered to *surgically* hepatectomised dogs, it does not accelerate the rate of fall of the blood-sugar, which it should do if it evoked a secretion of insulin in normal animals. Markowitz and Campbell [1927] found that when injected into a *functionally* hepatectomised dog, there was no evidence that the triose accelerated the rate of fall of the blood-sugar. However, this effect could have been masked by the presence of a slight amount of functioning liver tissue.

In favour of the insular origin of the hypoglycaemia following dihydroxyacetone may be mentioned the observation that the administration of the triose considerably augments the hyperglycaemia of a depancreatised dog. This finding, however, may possibly be dependent on the inability of the diabetic liver to hold glycogen, and not on the absence of insulin *per se.*

The ability of dihydroxyacetone to induce hypoglycaemia may be compared with the similar ability of glucose, under certain conditions, to bring about hypoglycaemia [Gibson and Larimer, 1924]. The administration of 50 g. glucose to a fasting normal human subject is regularly followed by hypoglycaemia after the initial hyperglycaemia has passed away. In some cases the hypoglycaemia is so profound as to cause symptoms like those of insulin overdosage. There is some evidence that the hypoglycaemia from glucose is dependent on an over-secretion of insulin. It is possible that dihydroxyacetone brings about hypoglycaemia in a similar fashion, although there is no evidence for this as yet.

(3) The liver is quite possibly concerned with the hypoglycaemia of dihydroxyacetone. Since the mechanism of the normal constant secretion of glucose into the blood by the liver is not known, the possible mechanism of this can only be vaguely conjectured. In our previous communication we put forward the tentative hypothesis that the extraordinarily high R.Q. following the administration of dihydroxyacetone was dependent on an increased tension of carbohydrate in the liver, an increase so great as not only to stop glyco-neogenesis out of fat and protein, but also to result in a considerable synthesis of fat. In the light of present knowledge it seems possible that a similar explanation may obtain in the case of the hypoglycaemic action

of this triose. Normally the cause for a greatly increased tension of carbohydrate in the liver is an excessive quantity of sugar of alimentary origin in the blood. Under these conditions it is possible that the hepatic secretion of blood-sugar ceases. When the tension of carbohydrate in the liver increases from any other cause, it is possible that an inhibition of the blood-sugar-secreting function of the liver ensues, which soon leads to hypoglycaemia, since the existing blood-sugar becomes rapidly used up.

In any case, there is no evidence for regarding the hypoglycaemic action of dihydroxyacetone in man as other than an adventitious one, being in this regard similar to glucose. It is therefore not dependent on any anti-diabetic qualities that the substance may possess. This viewpoint is in harmony with the fact that neither anti-diabetic nor hypoglycaemic functions of the triose have been demonstrable in the lower animals.

We should like to express our indebtedness to Miss E. L. Weatherhead for help with the blood-sugar determinations in these experiments.

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CLXXXVII. THE DIURNAL VARIATION OF THE GASEOUS CONSTITUENTS OF RIVER WATERS. PART II.

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(*Received November 7th, 1927.*)

The work described in Part I [1927] in connection with the diurnal variation of the gaseous constituents of river waters has been continued throughout the summer of 1927, and the following experiments over periods of 24 hours have now been performed.

River Lark.	Series	I.	March 30th–31st
	,,	II.	April 27th–28th
	,,	III.	May 18th–19th
	,,	IV.	June 28th–29th
	,,	IV *a*.	June 30th
	,,	V.	August 4th–5th
River Itchen.	Series	I.	April 6th–7th
	,,	II.	May 4th–5th
	,,	III.	May 31st–June 1st
	,,	IV.	July 5th–6th
	,,	V.	July 27th–28th

The results obtained in March and April on the River Lark and in April and May on the River Itchen have been given and discussed in Part I, while the remainder are here tabulated as follows:

Table	VI.	River Lark.	May 18th–19th.	Fig.	5
,,	VII.	,,	June 28th–29th.	,,	6
,,	VII (*a*).		June 30th.		
,,	VIII.	,,	August 4th–5th.	,,	7
,,	IX.	River Itchen.	May 31st–June 1st.	,,	8
,,	X.	,,	July 5th–6th.	,,	9
,,	XI.	,,	July 27th–28th.	,,	10

The times and values of the maxima and minima of the gaseous constituents on the River Lark are tabulated in Table XII, together with a summary of the weather conditions and river vegetation on the dates of the experiments.

Table VI. *River Lark. May* 18*th*–19*th*, 1927. *Series III.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
6 p.m.	15·0	1·696	165	0·01–0·015	8·0	Bright sun. Floating diatoms
7 ,,	14·5	1·567	151	0·01–0·015	8·0	Dull. Floating diatoms
8 ,,	14·25	1·451	139	0·01–0·015	8·0	Light failing
9 ,,	14·0	1·255	119·5	0·01–0·015	7·9–8·0	Dusk
10 ,,	13·4	1·099	104	0·01–0·015	7·8	Fine. Dark. Clear
11 ,,	13·2	0·977	92	0·015	7·7	Full moon rising. Fine
Midnight	13·0	0·824	77	0·015	7·6	Moonlight. Fine
1 a.m.	12·6	0·744	69	0·02	7·5–7·6	,, ,,
2 ,,	12·1	0·675	61	0·02–0·03	7·5–7·6	,, ,,
3 ,,	11·75	0·631	57	0·02–0·03	7·5	Sky lightening. Cloudy
4 ,,	11·6	0·519	47	0·08	7·5	Dull. Misty
5 ,,	11·0	0·419	37	0·16	7·5	Cloudy. Misty
6 ,,	11·0	0·407	36	0·16–0·18	7·5	Cloudy
7 ,,	10·8	0·431	38	0·16–0·18	7·5	Dull
8 ,,	10·8	0·448	40	0·16–0·18	7·6	Sun breaking through clouds
9 ,,	11·25	0·550	49	0·16–0·18	7·6	Bright sun
10 ,,	12·0	0·681	62	0·2	7·6	,,
11 ,,	12·0	0·894	81	0·18–0·2	7·6–7·7	,,
Midday	13·8	1·053	100	0·18–0·2	7·7	,,
1 p.m.	14·0	1·345	128	0·16	7·8–7·9	,,
2 ,,	14·6	1·569	151	0·08	7·8–7·9	,,
3 ,,	14·6	1·734	167	0·04	7·9–8·0	,,
4 ,,	15·0	1·851	180	0·03	8·0	,,
5 ,,	15·4	1·844	181	0·02	8·0	Cooler. Bright
6 ,,	15·2	1·822	177	0·01–0·015	8·0	,, ,,

Sunset May 18th, 7.48 p.m.
Sunrise ,, 19th, 4.4 a.m.

Table VII. *River Lark. June* 28*th*–29*th*, 1927. *Series IV.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
10 a.m.	12·6	0·618	57	0·16	7·5–7·6	Dull. Cloudy. Drizzle
11 ,,	12·75	0·682	63	0·12–0·13	7·6	,, ,, ,,
Midday	13·2	0·828	77	0·07–0·08	7·6	,, ,, ,,
1 p.m.	13·4	0·906	85	0·04	7·7	,, ,, ,,
2 ,,	14·2	1·033	98	0·025–0·03	7·7–7·8	Sun through
3 ,,	14·5	1·140	110	0·015	7·8	,,
4 ,,	14·3	1·153	111	0·015	7·8–7·9	Heavy rain. Dull
5 ,,	14·25	1·103	106	0·015	7·9	Drizzle. Rainstorm
6 ,,	14·2	1·063	102	0·01–0·015	7·9	Dull. Cloudy
7 ,,	14·1	1·073	102	0·01–0·015	7·9–8·0	,, ,,
8 ,,	13·8	1·033	98	0·01	7·9	Sunset
9 ,,	13·7	0·963	91	0·01–0·015	7·9	Dusk
10 ,,	13·5	0·886	84	0·01–0·015	7·8	Almost dark
11 ,,	13·5	0·824	78	0·01–0·015	7·7	Dark. Raining
Midnight	13·4	0·786	74	0·01–0·015	7·6–7·7	Clearer
1 a.m.	13·4	0·744	70	0·015	7·6	Fine. Cloudy
2 ,,	13·1	0·688	64	0·015–0·02	7·5–7·6	Sky just lightening
3 ,,	13·0	0·604	56	0·04	7·5	Light. Cloudy
4 ,,	12·8	0·489	45	0·08	7·5	Cloudy
5 ,,	12·8	0·427	40	0·13	7·4–7·5	Sun up
6 ,,	12·75	0·399	37	0·16	7·4–7·5	Sun through. Fine. Eclipse
7 ,,	12·75	0·420	39	0·16	7·4–7·5	Rain. Dull
8 ,,	12·7	0·482	45	0·16–0·18	7·4–7·5	,, ,,
9 ,,	12·7	0·514	48	0·18	7·5	Drizzle. Dull
10 ,,	13·1	0·590	55	0·16	7·5–7·6	Dull. Fine

Sunset June 28th, 8.21 p.m.
Sunrise ,, 29th, 3.45 a.m.
Sunset ,, 29th, 8.21 p.m.

Table VII (*a*). *River Lark. June* 30*th*, 1927. *Series IVa.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation of dissolved oxygen	Remarks
2 p.m.	15·3	0·93	91	Afternoon considerably more sunny than that of June 29th
3 „	15·4	1·004	98	
4 „	15·7	1·063	105	
5 „	15·9	1·114	110	
6 „	16·2	1·093	108	
7 „	15·9	1·091	108	
8 „	15·6	1·051	103	

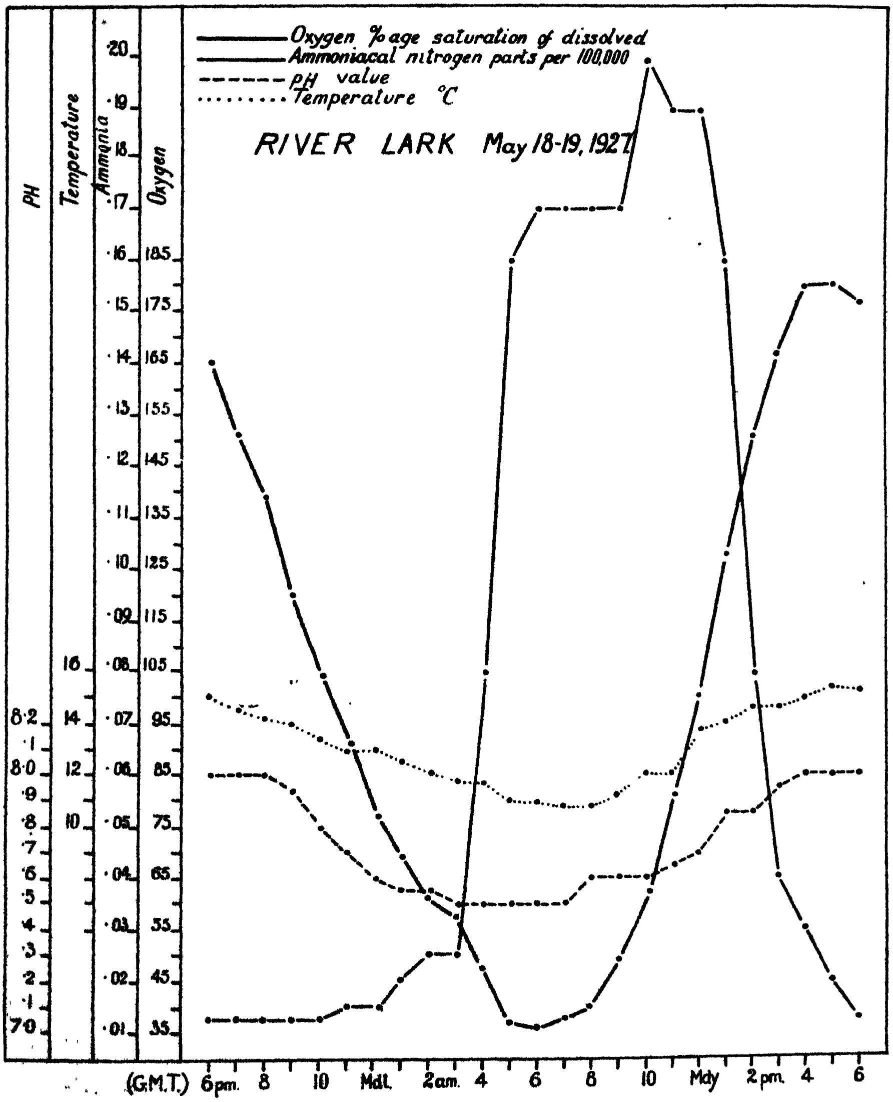


Fig. 5.

Table VIII. *River Lark. August 4th–5th,* 1927. *Series V.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 a.m.	16·25	0·579	58	0·01	7·5–7·6	Bright sun after bright morning
10 ,,	16·3	0·654	65	0·01	7·6–7·7	Bright sun
11 ,,	16·65	0·801	80	0·01–0·015	7·8	,,
Midday	17·6	0·971	99	0·015–0·02	7·9	,,
1 p.m.	17·9	1·079	111	0·015–0·02	8·0	Cloudy. Occasional sun
2 ,,	18·0	1·171	121	0·015–0·02	8·0–8·2	Cloudy. Water level falling rapidly. Mill stopped just before 2 p.m. and level rose again
3 ,,	18·1	1·148	118	0·015–0·02	8·0–8·2	Cloudy
4 ,,	18·2	1·174	121	0·015–0·02	8·0–8·2	Less cloud
5 ,,	18·4	1·197	124	0·015	8·0–8·2	Clear. Sunny
6 ,,	18·2	1·162	120	0·01–0·015	8·0–8·2	Sunny
7 ,,	17·85	1·060	109	0·01	8·0–8·2	Sun setting
8 ,,	17·6	1·038	106	0·01	8·0	Dusk. Fine
9 ,,	17·1	0·990	100	0·01	8·0	
10 ,,	16·9	0·891	90	0·01	7·8–7·9	
11 ,,	16·8	0·819	83	0·01	7·8	Clear
Midnight	16·8	0·772	77	0·01	7·7–7·8	,,
1 a.m.	16·35	0·708	71	0·01	7·7–7·8	Fine
2 ,,	16·2	0·678	67	0·01	7·6–7·7	Sky just lightening
3 ,,	16·1	0·603	60	0·01	7·6	Dawn
4 ,,	15·9	0·534	53	0·01	7·5–7·6	Daylight. Fine
5 ,,	15·8	0·493	49	0·01	7·5	Misty
6 ,,	15·8	0·458	45	0·01	7·5	,,
7 ,,	15·7	0·428	42	0·01	7·5	,,
8 ,,	15·8	0·442	44	0·01	7·5	Dull
9 ,,	16·1	0·528	53	0·01	7·5–7·6	Sunshine

Sunset August 4th, 7.45 p.m.
Sunrise ,, 5th, 4.28 a.m.
Sunset ,, 5th, 7.43 p.m.

Oxygen curves.

The oxygen curves of May, June and August are in general similar to those obtained in March and April, although the times of the maximum and minimum values vary to a certain extent.

One noticeable feature of the oxygen curves (R. Lark) is the progressive shortening of the period of minimum of oxygen from March (6 hours) and April (3 hours) to May (2 hours), while July and August give a single minimum value. These differences may be accounted for by the variation of the duration of the periods of darkness at the different dates.

Confirmatory results were not obtained on the Itchen since consistent small irregularities of samples were caused by the turbulence of this stream.

The time of the increase from the minimum value does not appear to be influenced by the time of sunrise so much as by the meteorological conditions.

It would appear that the current weather conditions have a considerable effect upon the times of the maximum and minimum values, and the results of an experiment performed during Series IV *a* on the River Lark bear out this point: on June 29th, after a dull day with intermittent rain, the maximum oxygen values (111 %, 110 %) were obtained at 3 and 4 p.m., whereas on the following day, June 30th, after an afternoon considerably more sunny than that of the previous day, the maximum values (110 %, 108 %, 108 %) were obtained from 5–7 p.m.

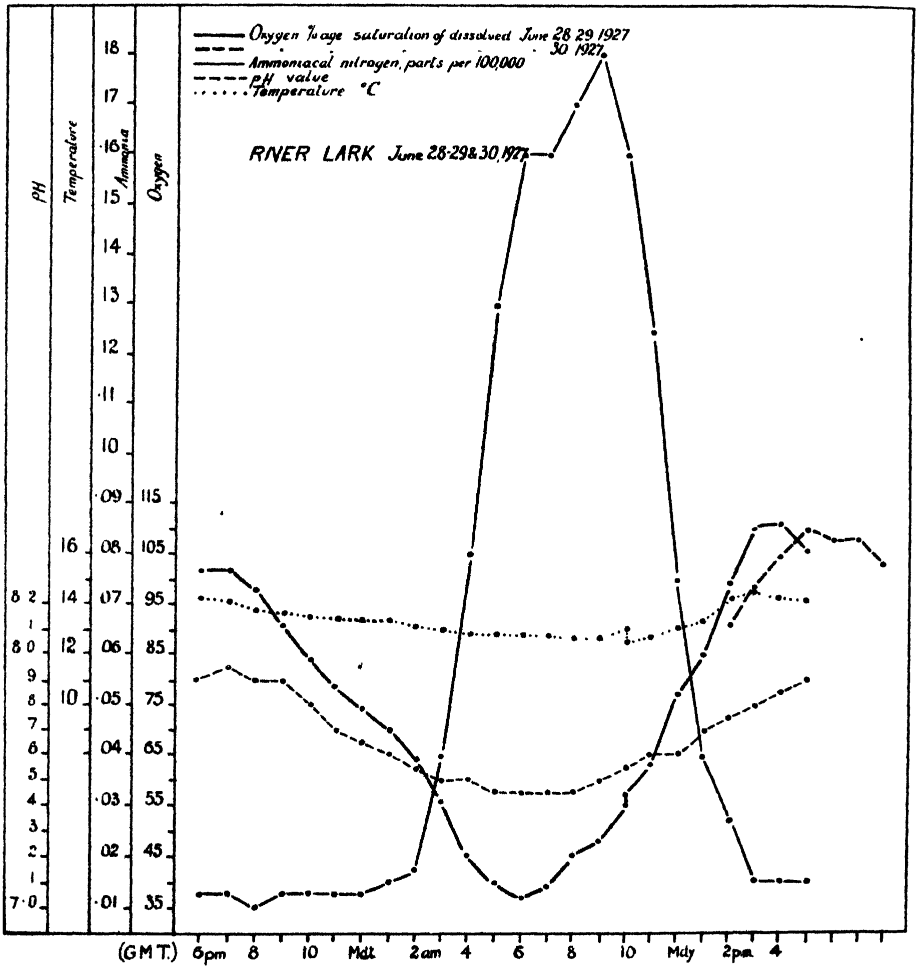


Fig. 6.

Considerable differences were noted in the maximum values of dissolved oxygen obtained on the River Lark; the March, April and May maxima were respectively 157, 178, and 181 % of saturation, and it is difficult at this stage to assign these differences definitely to the effects of either different actinic conditions or different conditions of plant life. The June and August maxima of 111 and 124 % respectively are considerably lower than those of March, April and May, and it is safe in conjunction with the botanical data (see p. 1433) to account for the difference by the decrease in the number of diatoms present.

Table IX. *River Itchen. May 31st–June 1st, 1927. Series III.*

Time G.M.T.	Temp. ° C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
10 a.m.	10·6	1·145	101	0·005	7·8	Dull showery. Water turbid
11 „	11·3	1·206	109	0·005	7·8	„ „
Midday	12·3	1·257	116	0·005	7·8	„ „
1 p.m.	12·5	1·172	109	0·005	7·8	„ „
2 „	12·8	1·245	116	0·005	7·9	„ „
3 „	13·5	1·192	112	0·005	7·85	„ „
4 „	13·9	1·135	108	0·005	7·85	Sunshine. „
5 „	14·3	1·201	115	0·005	7·85	„ „
6 „	14·0	1·115	106	0·005	7·85	„ „
7 „	13·9	1·103	105	0·005	7·8	„ „
8 „	13·0	1·004	94	0·005	7·8	Sunset. „
9 „	12·7	0·895	83	0·005	7·8	Cloudy. Almost dark
10 „	12·5	0·871	81	0·005	7·7	„ Dark
11 „	12·1	0·886	81	0·005	7·7	„ „
Midnight	12·0	0·827	76	0·005	7·7	„ Slight drizzle
1 a.m.	12·0	0·859	79	0·005	7·7	„ „
2 „	11·7	0·829	75	0·005	7·7	Cloudy. Slight drizzle. Sky lightening
3 „	11·5	0·852	77	0·005	7·6	Dawn. Slight drizzle
4 „	11·3	0·833	75	0·005	7·6	Dull. „
5 „	11·1	0·846	76	0·005	7·6	„ „
6 „	11·0	0·935	86	0·005	7·6	„ Fine
7 „	11·0	0·913	83	0·005	7·6	„ „
8 „	11·0	1·060	94	0·005	7·75	„ „
9 „	11·5	1·168	105	0·005	7·8	„ „
10 „	11·9	1·177	108	0·005	7·9	„ „

Sunrise May 31st, 3.50 a.m.
Sunset „ 31st, 8.5 p.m.
Sunrise June 1st, 3.49 a.m.

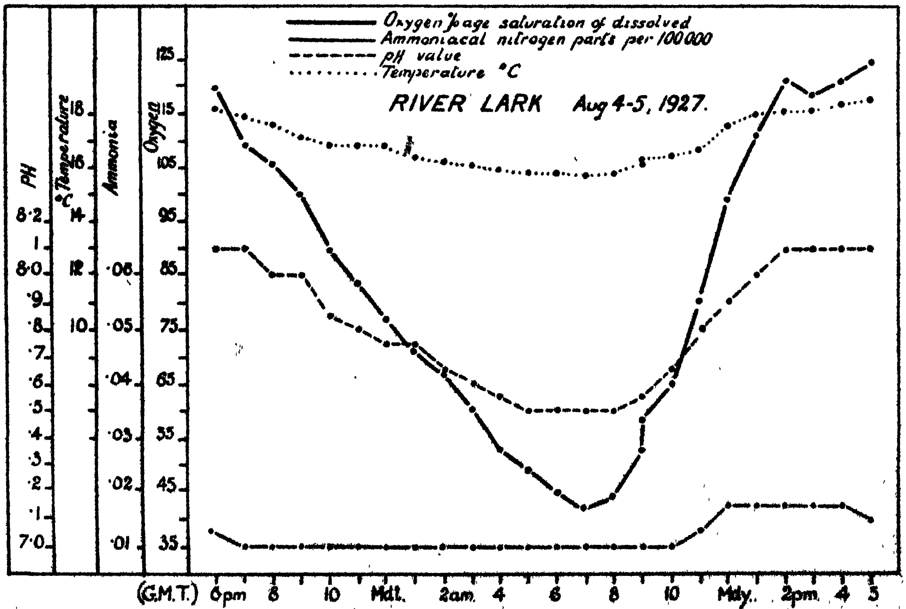


Fig. 7.

Table X. *River Itchen. July 5th–6th,* 1927. *Series IV.*

Time G.M.T.	Temp. °C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 a.m.	12·6	1·135	105	0·015–0·02	7·85	Thunderstorm 5.30–6.30 a.m.
10 „	13·1	1·151	108	0·005	7·9	Dull. Occasional sun
11 „	13·1	1·105	103	0·005	7·9	Dull. Raining. Windy
Midday	13·5	1·221	115	0·005	7·9	Dull. Fine
1 p.m.	13·6	1·147	108	0·005	7·9	„ „
2 „	14·1	1·271	121	0·005	7·95	„ „
3 „	15·0	1·217	118	0·005	8·0	Brighter.
4 „	15·0	1·203	117	0·005	8·0	„ Little sun
5 „	15·0	1·200	116	0·005	8·0	Sunshine
6 „	15·1	1·167	113	0·005	8·0	„
7 „	14·9	1·122	109	0·005	8·0	„
8 „	14·0	0·998	95	0·005	7·85	„ Sun low
9 „	13·0	0·923	86	0·005	7·7	Dusk. Fine. Moon shining
10 „	12·5	0·932	86	0·005	7·7	Clear. „ „
11 „	12·0	0·857	79	0·005	7·6	„ „
Midnight	11·8	0·880	80	0 005	7·5	Slight cloud. Moonset
1 a.m.	11·6	0·825	74	0·005	7·5	Cloudy. Sky just lightening
2 „	11·5	0·838	76	0·005	7·5	Daybreak. Some cloud. Fine
3 „	11·1	0·807	72	0·005	7·5	Dawn. Some cloud. Fine
4 „	11·0	0·838	76	0·005	7·5	Daylight. Fine. Clear
5 „	10·7	0·893	79	0·005	7·5	Sunshine
6 „	10·9	0·919	82	0·005	7·6	„
7 „	11·2	1·026	92	0·005	7 7	„
8 „	12·0	1·154	106	0·005	7·8	„ Wind rising
9 „	12·6	1·177	109	0·005	7·9	Dull. Fine

Sunrise July 5th, 3.49 a.m.
Sunset „ 5th, 8.19 p.m.
Sunrise „ 6th, 3.50 a.m.

It would appear that the oxygen exuded from diatoms is more likely to produce a supersaturated solution than is that evolved from the higher green plants which tends to escape in bubbles from their relatively large stomata.

The minimum oxygen values in May, June and August are considerably lower than those of March and April, and it is possible that the larger green plants present during the summer (which were absent in March and April), by increased respiration, have caused this decrease in oxygen during the night. This is confirmed by the slightly lower p_H values obtained during the nights of May, June and August compared with those of March and April. An added factor to the progressive decrease in value of the oxygen minima may be the increased oxygen-consuming power of the river bed and vegetation due to the seasonal increase in temperature.

Nitrogen curves.

As in Series I and II on the River Itchen, no diurnal variation of ammoniacal nitrogen was recorded in Series III, IV and V.

On the River Lark the ammoniacal nitrogen curves of May and June are in general similar in structure to those of March and April, although considerable differences were obtained in the times of the maximum periods. The maximum periods of March and April were from 2–5 a.m. and from

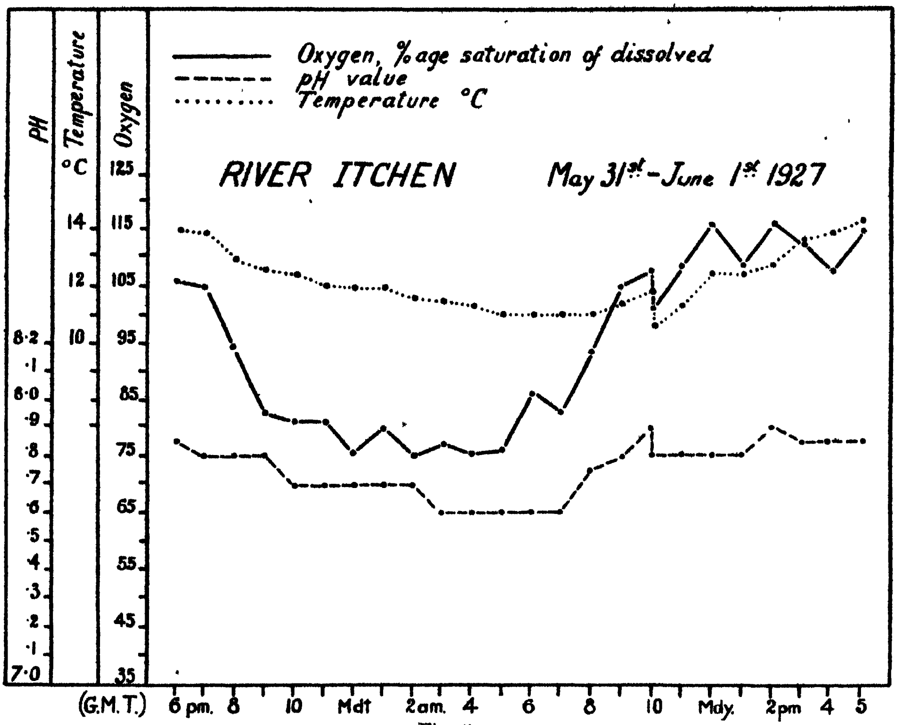
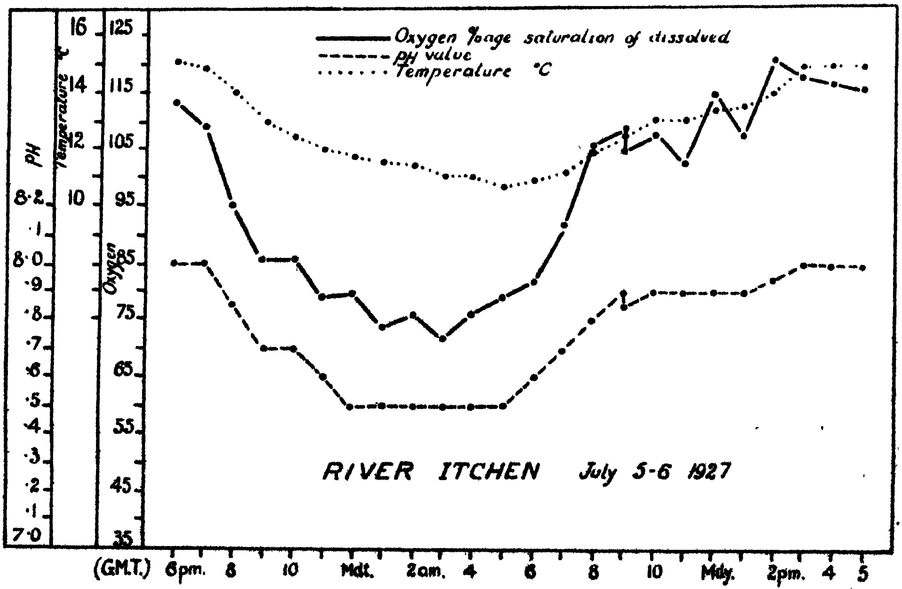

Oxygen, %age saturation of dissolved
pH value
Temperature °C
RIVER ITCHEN
May 31st - June 1st 1927
PH
Temperature °C
Oxygen
8·2
·1
8·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
7·0
14
12
10
125
115
105
95
85
75
65
55
45
35
(G.M.T.)
6 pm.
8
10
Mdt
2am.
4
6
8
10
Mdy.
2pm
4
5

Fig. 8.


Oxygen %age saturation of dissolved
pH value
Temperature °C
RIVER ITCHEN
July 5-6 1927
PH
Temperature °C
Oxygen
8·2
·1
8·0
·9
·8
·7
·6
·5
·4
·3
·2
·1
7·0
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14
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125
115
105
95
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75
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55
45
35
(GMT.)
6pm.
8
10
Mdt.
2am.
4
6
8
10
Mdy.
2pm.
4
5

Fig. 9.

Table XI. *River Itchen. July 27th–28th*, 1927. *Series V.*

Time G.M.T.	Temp. °C.	Dissolved oxygen. Parts per 100,000	% saturation dissolved oxygen	Ammoniacal nitrogen. Parts per 100,000	p_H value	Remarks
9 a.m.	12·8	1·060	99	0·005	7·8	Much rain 4–6 a.m. Bright occasional sun. River turbid
10 „	13·5	1·122	106	0·005	7·9	Cloudy. Windy
11 „	13·9	1·128	107	0·005	7·9	Sunshine. Water clearing
Midday	14·7	1·241	119	0·005	8·0	Cloudy. Windy. Water clear
1 p.m.	15·4	1·223	120	0·005	8·1	Sunshine. Windy
2 „	16·0	1·270	126	0·005	8·2	Cloudy. „
3 „	15·9	1·1825	117	0·005	8·2	„ „
4 „	15·5	1·193	117	0·01	8·2	Dull. Very rough
5 „	15·0	1·107	107	0·005	8·0	„ Windy
6 „	14·2	1·067	102	0·005	7·9–8·0	„ Raining 5–6 p.m. Windy
7 „	13·6	0·970	91	0·005	7·9	„ „ „
8 „	13·1	0·863	81	0·005	7·8	Fine. Dull. Windy
9 „	12·9	0·821	77	0·005	7·7	Almost dark. Windy. Cloudy
10 „	12·5	0·833	75	0·005	7·6–7·7	Dark. Windy. Raining
11 „	12·1	0·808	74	0·005	7·6	Dark. Wind dropped. Raining
Midnight	12·0	0·784	72	0·005	7·5	Wind rising. Fine. Cloudy
1 a.m.	12·0	0·789	72	0·005	7·5	Some wind. „ „
2 „	12·0	0·801	73	0·005	7·5	Rain 1.15 a.m. „ „
3 „	12·0	0·760	68	0·005	7·5	Sky lightening. Less wind
4 „	11·8	0·801	73	0·005	7·4–7·5	Dawn. Fine. Some cloud
5 „	11·9	0·793	73	0·005	7·5	Daylight. Cloudy
6 „	12·1	0·834	77	0·005	7·6	Dull. Fine. Some wind
7 „	12·7	0·931	86	0·005	7·7	„ „ Sunny intervals
8 „	13·3	1·106	104	0·005	7·9	„ „
9 „	13·4	1·234	116	0·005	7·9–8·0	„ „

Sunrise July 27th, 4.14 a.m.
Sunset „ 27th, 7.58 p.m.
Sunrise „ 28th, 4.16 a.m.

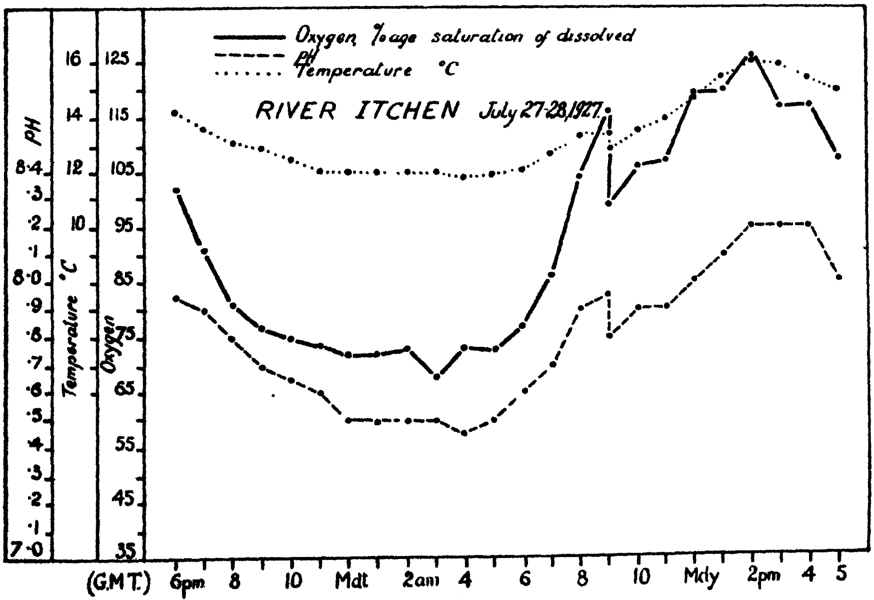

Fig. 10.

Table XII.

Date	% saturation of dissolved oxygen		p_H value		Ammoniacal nitrogen		Temperature ° C.		Weather conditions	Sunrise a.m.	No. of days from June 21st	Plants
	Max.	Min.	Max.	Min.	Max.	Min.	Max.	Min.				
March 30th–31st	3 p.m.	1–7 a.m.	2–7 p.m.	2–9 a.m.	2–5 a.m.	3–6 p.m.	3–4 p.m.	5 a.m.				Submerged and littoral plants just appearing. Diatoms abundant
	157	65	7·9–8·0	7·6–7·7	0·16	0·01	10·0	6·75	Bright	5·40	82	
April 27th–28th	3–4 p.m.	3–6 a.m.	2–6 p.m.	4–7 a.m.	3–9 a.m.	4–11 p.m.	6 p.m.	5–7 a.m.				Submerged and littoral plants 1 ft. in height. Diatoms abundant. Green algae appearing
	178	57	8·0	7·5–7·6	0·16	0·02	11·5	7	Sunny	4·40	55	
May 18th–19th	4–5 p.m.	5–7 a.m.	4–8 p.m.	3–7 a.m.	6 a.m.–midday	6–10 p.m.	5 p.m.	7–8 a.m.				Submerged plants at height of growth. Diatoms on decrease. Green algae dominant
	181	36	8·0	7·5	0·2	0·01–0·015	15·4	10·8	Sunny	4·5	35	
June 28th–29th	3–4 p.m.	6 a.m.	5–9 p.m.	5–8 a.m.	6–10 a.m.	6 p.m.–midnight	3 p.m.	8–9 a.m.				Submerged plants at height of growth. Cut June 22nd. Few diatoms. Some green algae
	111	37	7·9–8·0	7·4–7·5	0·18	0·01	14·5	12·7	Dull and rain	3·40	8	
June 30th	5–7 p.m.	—	—	—	—	—	6 p.m.	—	Afternoon more bright than June 29th			
	110	—	—	—	—	—	16·2	—				
August 4th–5th	4–5 p.m.	7 a.m.	2–7 p.m.	5–8 a.m.	1–4 p.m.	7 p.m.–10 a.m.	5 p.m.	7 a.m.				Submerged plants at height of growth. Some green algae
	124	42	8·0–8·2	7·5	0·015–0·02	0·01	18·4	15·7	Sunny	4·20	44	

3–9 a.m. respectively, whereas those of May and June were from 6 a.m.–midday and from 6–10 a.m. In this respect, some slight relation is shown between the curves of ammoniacal nitrogen and temperature, and it would appear that the higher afternoon temperature of May over June corresponds to an increased and later production of ammonia in May compared to that of June.

A remarkable difference was noted in the ammoniacal nitrogen curve obtained on August 4th–5th—in this case only small differences were obtained throughout the 24 hours, and the maximum period took place between 1 and 4 p.m.

It is difficult to account for this difference between the August curve and those of the previous months, but it is most probable that the river flora and the condition of the river bed are the dominant factors in the disappearance of the high nitrogen values obtained in Series I–IV.

p_H *curves.*

The May, June and August curves were similar in structure to those of March and April, and in general again follow the oxygen curves.

Temperature curves.

All the temperature curves show the expected diurnal similarity, but over different ranges.

VEGETATION OF THE RIVERS.

A detailed account is given below of the vegetation on the Rivers Lark and Itchen at the times of the various experiments.

The River Lark. For 2 miles above the point of sampling at Icklingham the river is slow flowing and moderately silted. In the summer there is a broad margin of erect littoral plants, mostly *Sparganium erectum* and *Glyceria aquatica*. The submerged plants are sufficiently abundant to hold up the water, causing flooding of the adjoining land even at dry periods. A mile above the point of sampling, the dominant plants are *Potamogeton lucens* and *P. pectinatus*, while immediately above the station, where the water is somewhat deeper, are *Sagittaria sagittifolia* and *Sparganium simplex*. The following is a full account of the vegetation at the various dates of the experiments.

March 30th. The submerged plants were just appearing above the mud and were about 2 inches high. The littoral plants were also just appearing through the mud and previous year's debris.

The diatoms were very abundant, and the figures from deposits on glass slides are given below.

March 9th to 23rd	(1)	10 mg./cm.^{2}
	(2)	15 ,,
March 23rd to April 7th	(1)	73 ,,
	(2)	70 ,,

Dominant organisms: *Synedra ulna* (about 50 %), *Nitzschia acicularis, Navicula Reinhartii, N. viridula.*

April 27*th*. The submerged and littoral plants were about 1 foot in height but vigorous growth had not commenced. The diatoms were still abundant, but the proportions of species were not identical.

Deposits:	April 7th to 21st	(1)	47 mg./cm.^{2}
	April 21st to May 5th	(2)	55 ,,

Dominant organisms:

(1) *Synedra ulna* 40 %, *Gomphonema olivaceum* 35 %, *Nitzschia acicularis, Diatoma vulgare, Melosira varians.*

(2) *Synedra ulna* 40 %, *Ulothrix zonata* 30 %, *Stigeoclonium tenue, Gomphonema olivaceum, Nitzschia acicularis, Amphora minuta.*

A point of note in Sample (2) is the presence of two filamentous green algae among the diatoms.

May 18*th*. The submerged vegetation had now reached its most vigorous stage of growth and was beginning to hold up the water. Just above the sampling station there was a considerable amount of *Sparganium simplex* (a grass-like plant) but the dominant plants were *Potamogeton lucens* and *P. pectinatus*, both of which were overgrown with *Cladophora glomerata*, a filamentous green alga.

Diatom deposits: May 14th to 30th, 45 mg./cm.^{2}

Dominant organisms: *Ulothrix zonata* 70 %, *Synedra ulna, Cymbella gastroides, Amphora minuta.*

The diatoms were definitely on the decrease and the filamentous green algae were dominant.

June 29*th*. Towards the end of May the weeds grew very rapidly, causing flooding of the adjoining fields. On June 22nd weed cutting took place at Icklingham, so that on June 29th a normal amount of vegetation was present, chiefly *Potamogeton lucens* and *Sparganium simplex.* Two miles above the sampling station the littoral plants were fully grown.

Diatom deposits:

May 30th to June 16th	(1)	1·2 mg./cm.^{2}
	(2)	2·3 ,,
June 16th to 30th	(1)	1·2 ,,
	(2)	2·4 ,,

Dominant organisms: *Chlorococcum infusionum* and *Protoderma viride*; the deposit consisted of 80 % of *Protococcales.* There were few diatoms; *Amphora minuta, Synedra ulna, Cymbella gastroides.*

August 3*rd*. The submerged plants were very abundant, and there was more *Sparganium simplex* than in June. *Cladophora glomerata* and *Potamogeton pectinatus* were present in smaller quantity.

Deposits:	July 1st to 18th	(1)	1·1 mg./cm.^{2}
		(2)	0·7 ,,
	July 18th to August 3rd	(1)	1·4 ,,
		(2)	1·2 ,,

Dominant organisms: *Protoderma viride* and *Chlorococcum infusionum.*

The River Itchen. The Itchen at the Alresford Research Station is a swift stream and the dominant plants are *Sium angustifolium* and *Ranunculus penicillatus*, although immediately above the weir where the samples are taken is a short silted stretch (about 50 yards) with *Callitriche stagnalis* and *Potamogeton densus* in some quantity. Littoral plants are scarce and consist of plants of the habit of *Nasturtium officinale* (water-cress) and *Myosotis palustris* (forget-me-not).

The most remarkable feature of the phanerogamic vegetation is that it persists throughout the year, the area of green plants in the water being not much less in December than in July. The area occupied by the plants is also small in comparison with that in the Lark. The increase in the vegetation therefore in the period considered may be taken as small.

The micro-flora. No direct measurements or systematic collections have been made of the diatoms on the Itchen this year but from previous collections the general seasonal variation is the same as for the Lark, though the quantities are not so large. There is a maximum growth of diatoms in March and April, the dominant organisms being *Synedra ulna*, *Gomphonema olivaceum* and *Amphora minuta*. These decrease in May and are followed by the appearance, as small tufts on all the stones, of the filamentous algae, *Vaucheria* sp. and *Cladophora* sp. At the end of August these had disappeared and all the micro-flora there was consisted of a few diatoms, *Synedra*, *Gyrosigma*, etc. and *Batrachospermum* in comparatively small quantity.

In conclusion the authors wish again to thank Sir R. Robertson, the Government Chemist, and the Ministry of Agriculture and Fisheries, for permission to publish these results.

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